COMMUNAUTE FRANCAISE DE BELGIQUE
ACADEMIE UNIVERSITAIRE WALLONIE-EUROPE
UNIVERSITE DE LIEGE – GEMBLOUX AGRO-BIO TECH

Chemical ecology in earthworms: food foraging strategy and intra-specific interactions in *Eisenia fetida* (Savigny, 1826) and potential applications in vermicomposting

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Essai présenté en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

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2012

Zirbes Lara M. (2012). Chemical ecology in earthworms: food foraging strategy and intra-specific interactions in *Eisenia fetida* (Savigny, 1826) and potential applications in vermicomposting (PhD thesis). Gembloux, Belgium, University of Liege, Gembloux Agro-Bio Tech, 105 p., 6 tabl., 14 fig.

Abstract – The volume of domestic and industrial wastes is greatly increasing across the world, in turn increasing demand for biological methods to minimize environmental pollution. Earthworms could provide part of this package of biological solutions, by means of vermicomposting processes. More specifically, *Eisenia fetida* are the earthworm species most commonly used for organic waste management by vermicomposting.
The main objective of this thesis was to investigate foraging strategy and intra-specific interactions in compost earthworms *E. fetida* using chemical communication, a new approach in earthworm ecology studies. Our main findings were as follows. Firstly, using olfactometer assays combined with chemical analyses (GC-MS), we documented the attraction of *E. fetida* individuals to filtrate derived from *Geotrichum candidum* colonies, and also to two individual compounds tested in isolation: ethyl pentanoate and ethyl hexanoate. Attraction at a distance was observed when barriers prevented the worms from reaching the target stimuli, confirming the role of volatile cues. Secondly, we have shown that *E. fetida* earthworms influence each other to select a common direction during their migration. Experiments in a binary choice test showed that contacts between individuals are responsible for collective movement. This coordination in movement could allow earthworms to benefit by forming clusters. We have also documented the ability of *E. fetida* individuals to join and leave a cluster. We have identified, for the first time, self-assembled structures in earthworms and clarified the mechanisms of cluster formation. Attraction at a distance was observed, suggesting the role of volatile cues in cluster formation. In addition, we have found that the survival of the cluster was dependent on the initial population size. This survival presents an all-or-one phenomenon or quorum, confirming the importance of a great number of earthworms in the cluster for its stability. These findings enhance our understanding of the mechanisms underlying key trophic interactions in soil ecosystems, as well those involved in cluster and collective movement in earthworms. The findings also have potential implications for the extraction and collection of earthworms in vermiculture and other applied activities.

Zirbes Lara M. (2012). Ecologie chimique des vers de terre: stratégie de recherche de nourriture et interactions intra-spécifiques chez *Eisenia fetida* (Savigny, 1826) et les applications potentielles dans le lombricompostage (Thèse de doctorat). Gembloux, Belgium, University of Liege, Gembloux Agro-Bio Tech, 105 p., 6 tabl., 14 fig.

Résumé - L'accumulation des déchets industriels et ménagers ne cesse d'augmenter, entrainant le développement de méthodes biologiques qui minimisent la pollution environnementale. Le ver de terre apparaît être un excellent candidat pour cette tâche au travers du lombricompostage. Plus particulièrement, *Eisenia fetida* est une espèce de vers de terre communément utilisée dans la gestion des déchets par lombricompostage. Dans ce contexte, l'objectif principal de cette thèse de doctorat était d'explorer les stratégies de foraging et les relations intra-spécifiques chez le ver de terre du compost, *E. fetida*, au moyen de la communication chimique comme nouvelle approche pour les études sur l'écologie des vers de terre. Premièrement, à l'aide d'essais olfactométriques combinés aux analyses chimiques (GC-MS), nous avons montré l'attraction d'*E. fetida* pour le filtrat dérivé des colonies de *Geotrichum candidum* et à deux composés testés individuellement : le pentanoate d'éthyle et l'hexanoate d'éthyle. De l'attraction à distance a été observée lorsque des barrières empêchaient les vers d'atteindre les stimuli, confirmant le rôle de signaux volatils. Deuxièmement, nous avons montré que les vers de terre *E. fetida* s'influencent les uns les autres pour choisir une direction commune durant leur migration. Les expériences en choix binaire ont montré que le contact entre individus était responsable des mouvements collectifs. Cette coordination de mouvements pourrait permettre aux vers de terre de former des agrégats statiques. Nous avons également montré la capacité d'*E. fetida* de joindre et de quitter un agrégat. Nous avons identifié, pour la première fois, des structures auto-assemblées chez les vers de terre et clarifié les mécanismes de formation des agrégats. De l'attraction à distance a été observée, suggérant le rôle de signaux volatils dans la formation des agrégats. De plus, nous avons trouvé que la survie des agrégats dépendait de la taille de la population initiale. Cette survie présente un phénomène de tout ou rien ou de quorum, confirmant l'importance du grand nombre de vers de terre dans la stabilité de l'agrégat. Ces découvertes améliorent notre compréhension des mécanismes soulignant les interactions trophiques dans les écosystèmes du sol, et ceux impliqués dans les agrégats et les mouvements collectifs des vers de terre, et ont des applications potentielles dans l'extraction et l'échantillonnage des vers en lombriculture et dans d'autres activités appliquées.

I want to thank everyone who contributed scientifically or not in the realization of this thesis. My thanks are especially addressed to

- the professor Eric Haubruge, promoter of this PhD thesis, to welcoming me in his department, .for his encouragements, for his many advices, ideas and guidelines in my researches but also to give me the opportunity to discover Vietnam;
- the professor Philippe Thonart, co-promoter of this PhD thesis, to his closed collaboration concerning fungus colonies production and for his valuable suggestions on my research;
- the professor Jean-Louis Deneubourg for our long discussions about earthworm behaviour, for all data analyses, simulations and interpretations enhancing considerably my results;
- the professors George Lognay, Jean-Paul Wathelet, and Frederic Francis and mister Gilles Colinet for their valuable suggestions and contributions;
- Mister François Verheggen for many advices on chemical ecology and in the drafting and the English of articles related to the thesis;
- the professor Charles Gaspard predestined to carry out this thesis by ask me some questions on earthworms in 1st candi tests;
- the whole team of the department of functional and evolutionary entomology. Special thanks to Kim and Jess, my office team, for giggling, for your encouragements, your motivations and all good time shared. Special thanks also to Didier for his technical assistance, to Marceline and Jeannine for all their attentions;
- the bachelor students who contributed directly to this work: Vladimir Fanali, Véronique Vrancken, Maxime Jason, and Jeremie Deprez;
- the financial support of the "Fond pour la formation à la recherche dans l'industrie et dans l'agronomie" that helped me to conduct my PhD thesis;
- all my friends to try to understand how I can study earthworms and their communications, and to think of me when they find an earthworm in their gardens;
- my parents and my brother for their encouragements throughout my thesis;
- my husband Seb, who was by my side along my entire PhD thesis, for his patience and his encouragements. A special thank to my baby Thibault.

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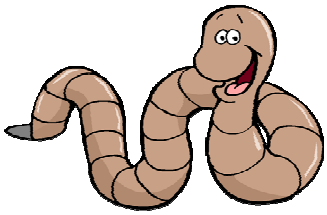
Chapter 1: General introduction



Earthworms (Oligochaeta, Lumbricidae) are undoubtedly the major components of soil biota in terms of soil formation as well as maintenance of soil structure and fertility (Edwards, 2004). The importance of earthworms in the breakdown of organic matter and the associated release of nutrients has been long accepted (Darwin, 1881). Domestic and industrial waste is greatly increasing, on a global scale, creating an increased demand for biological methods to minimize environmental pollution (Edwards and Bohlen, 1996). Earthworms appear to be an excellent candidate for this task, and vermicomposting processes have already been developed (Magdoff and Weil, 2004). Indeed, it has been clearly demonstrated that some earthworm species are specialized to live in decaying organic matter and can degrade it into fine particulates, a behaviour with considerable commercial potential as a plant growth media or soil amendment due to its richness in available nutrients (Dominguez, 2004). In temperate regions, *Eisenia fetida* are the earthworm species most commonly used for organic waste management by vermicomposting. These earthworms are interesting candidates because *E. fetida* are a ubiquitous species with a worldwide distribution, naturally colonizing many organic wastes (e.g. Reneicke and Viljoen, 1990; Dominguez and Edwards, 1997). They have good humidity and temperature tolerances and they are well adapted to human manipulation. Under optimal condition, their short cycle life range (from 45 to 51 days) creates further commercial opportunities in the supply of earthworms for industrial vermicomposting (Edwards and Bolhlen, 1996).

Although there are an increasing number of publications on earthworms in fields of biology and ecology (Edwards, 2004), there is nonetheless an urgent need for greatly expanded research, particularly on some specific aspects of earthworms' activities. Indeed, very few studies have addressed the questions of the detailed interrelationships among earthworms, microorganisms and decaying organic matter whereby interactions between organic matter and microorganisms provide food for earthworms. Moreover, nothing is known about the chemical ecology of earthworms, whereas extensive research has documented the role of olfactory cues in mediating above-ground ecological interactions among plants, microbes, and invertebrates. Semiochemicals are volatile and therefore easy to disperse and detect: recent work suggests that such cues play similarly important and complex roles in below-ground interactions (Laothawornkitkul et al., 2009; Wenke et al., 2010), but we are only beginning to understand the chemical ecology of these systems. Earthworms are known to possess chemoreceptors (Edwards and Bohlen, 1996) and previous studies have demonstrated their ability to orient and move toward microbial food sources, but the specific

chemical cues guiding earthworm foraging have not previously been documented. We also lack knowledge about the intra-specific interactions of earthworms. We can use the new information gained from our research to reanalyze the spatial organization of earthworms, as well as the soil dynamics such as their formation, fertility or bioremediation.

In this context, the overall objective of this thesis was to investigate the food foraging strategy and intra-specific interactions in compost earthworms, *Eisenia fetida*, using chemical communication, which is a new approach in earthworm ecology studies. In addition to elucidating the sensory cues that guide earthworm feeding behaviour and contribute to cluster formation, the potentially important implications for vermicomposting and other agricultural applications are considered.

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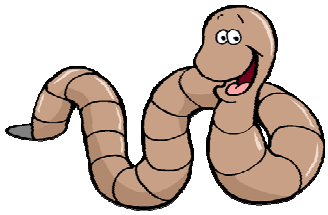
Chapter 2: Objectives



The aim of this thesis was to investigate foraging strategy and intra-specific interactions in compost earthworms, *Eisenia fetida*. More specifically, the first objective was to explain how earthworms select and store a specific food resource. Previous studies have demonstrated that earthworms do not feed at random, but rather display food preference. The second objective focused on the mechanisms implicated in cluster formation and collective movement in earthworms, two social behaviours widely observed in many biological systems ranging from microorganisms to humans. To achieve these objectives special attention was paid to chemical communication, which is commonly observed in inter- and intra-interactions in various phyla including arthropods and nemathelminthes.

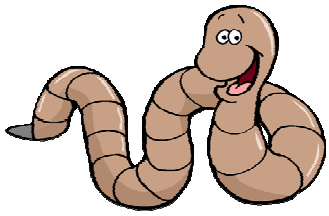
In the third chapter of this thesis, earthworm behaviour was more closely observed in order to identify responses to cues associated with the soil fungus *Geotrichum candidum*, an important food source for *E. fetida*. With the hypothesis that olfactory cues from a food source could act as semiochemicals for *E. fetida*, we wanted to identify and to test these semiochemicals as potential attractant. The objective of this study was to explore the implications of volatile cues for the food foraging behaviour of earthworms. No previous study has dealt with chemical communication in earthworms whereas soil and microorganisms were considered as volatile producers.

Preliminary observations suggested intra-specific (social) interactions in *E. fetida*. In the fourth chapter, we wanted to understand how unexpected observed self-assemblages of earthworms are formed. The dynamics of aggregation survival depends on the probability of individuals leaving an aggregate and/or the probability of joining it; therefore these two mechanisms were studied with the hypotheses that both chemical cues and density dependence are implicated in the clustering phenomenon.

In this chapter, we also wanted to explore the possibility that earthworms make consensual decisions; more specifically, we wanted to establish the degree of potential collective movement and to investigate whether chemical and physical cues are involved in this particular behaviour.

The last part of this thesis comprises a more general discussion of the results obtained and formulates perspectives for additional experiments that could further enhance our knowledge of chemical communication between, and behaviour of, earthworms, *Eisenia fetida*.

Chapter 3: Food foraging strategy in Eisenia fetida (Savigny, 1826)



3.1. General introduction

Interactions between earthworms and microorganisms are important for soil processes such as decomposition and transformation of plant residue, humus formation, and the formation of the pool of nutrient elements and microbial communities. The wide spectrum of these interactions infers a close relationship between earthworms and microorganisms. Fungi and bacteria are assumed to be the main source of food for the earthworm. As previously mentioned, food preference experiments have shown that earthworms do not feed at random: some microbes are preferentially ingested by earthworms, while others are rejected. Previous studies exploring the feeding strategies of various earthworm species suggest that these animals exhibit orientation and movement toward particular food sources, including specific species of protozoa, bacteria, fungi and plants. However, previous studies have not explicitly documented the cues responsible for food preference.

Microorganisms form both a major component of earthworm diets, and a principale source of volatile organic compound emissions in soil ecosystems, suggesting that olfaction may play a key role in earthworm foraging. Indeed, chemoreceptors have been identified on the prostonium and the buccal epithelium of earthworms and have been shown to detect sucrose, glucose and quinine.

In this chapter, we intend to summarize the present knowledge of microscale interactions between earthworms and microorganisms in soil and then we focus our attention on one particular microscale interaction: food preference. Indeed, we explored the hypothesis that earthworms, *Eisenia fetida*, use volatile chemicals associated with the soil fungus *Geotrichum candidum* (one of their primary food sources) during food foraging.

3.2. Microscale interactions between earthworms and microorganisms

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Reference: Lara Zirbes, Philippe Thonart, and Eric Haubruge (2011) Microscale interactions between earthworms and microorganisms. Biotechnologie, Agronomie, Société et Environnement (Accepted)

Abstract – Microorganisms are well adapted to their soil microhabitat where they live together in consortia, interacting with other living members, including earthworms. This literature review consists of four sections that focus on microscale interactions between earthworms and microorganisms. The first part is devoted to nephridia symbiosis. Recent discoveries show that *Verminephrobacter* spp. is present as a symbiont in earthworm nephridia. The second section deals with earthworm food preference and focuses on the major hypotheses of foraging strategies. The third section presents evidence of gut symbionts and highlights the need for additional studies in this field. The last section of this review explains why microorganism activities are enhanced in burrows and casts of earthworms.

1. Introduction

Few soil ecology studies are focused on the prospects of linking microbes and fauna (Brown et al., 2004; Coleman et al., 2004). Without doubt, earthworms are the most important soil invertebrates in the soil ecosystem, in terms of biomass and activity (Römbke et al., 2005), being often considered as ecosystem engineers (Lavelle, 1988). Moreover, soil contains a large diversity of microorganisms (Torsvik et al., 2002). Microorganisms are an unavoidable constituent of earthworms' natural diet. Three earthworm ecological groups are generally defined and earthworms feeding behaviour is clearly associated to their ecological group (Bouché, 1977; Lee, 1985; Edwards et al., 1996; Brown et al., 2000):

- Epigeic: Earthworm species belonging to the Epigeic group live on or near the soil surface, typically in the litter layers of forest soils and do not burrow. Epigeic earthworms consume plant litter and litter inhabiting organisms and ingest little or no soil. They are qualified as "litter transformers" (Lavelle et al., 1997). Several studies have shown that epigeic activities induce an increase in microbial activities due to greater surface area for decomposition, reduce immobilization by surface-litter dwelling fungi and modify the composition of microorganism communities (Scheu et al., 1994; Parkinson et al., 1998).
- Anecic: Earthworms from the Anecic group live in permanent or semi-permanent vertical burrows in the mineral soil layers. Anecics feed on organic matter mixed with soil particles, bury surface litter, and often forming middens. Middens consist of an accumulation of surface castings mixed with organic matter in which microorganisms multiply and microbial degradation of uningested organic matter is improved (Brown, 1995; Maraun et al., 1999; Tiunov et al., 2000; Shuster et al., 2001). The amount of ingested organic matter by anecics varies with its quality (e.g. nitrogen, lignin or tannin quantities or C/N ratio) and its microbial composition. Anecic earthworms seem to prefer litter rich in nitrogen, without tannins or colonised by particular fungi species including *Fusarium lateritium* Nees and *Trichoderma* sp. (Cooke et al., 1980; Moody et al., 1995).
- Endogeic: these earthworms typically live in mineral soil horizon, making horizontal burrows mainly at a depth of 10-15cm. They consume more soil than other ecological categories and are often called soil organic matter feeders or geophageous.

Interactions with microorganisms are observed in earthworm burrow lining, cast, nephridia or gut, in the drilosphere (Brown et al., 2004). Drilosphere is the soil area directly or indirectly influenced by earthworms' activity and is constantly changing in space and time (Lavelle, 1988). Together, earthworms and microorganisms mineralise, humify organic matter and facilitate chelation of metal ions (Lavelle et al., 1995; Cai et al., 2002). Microorganisms help

earthworms in their growth (Pizl et al., 2003). For example, Miles (1963) observed that *Eisenia fetida* Savigny hatched from microbiologically sterile cocoons could not reach sexual maturity in sterilised soil until mixed cultures of mobile protozoa were added in its food. Growth and reproduction in earthworms require carbon and nitrogen coming from litter, grit and microorganisms (Edwards et al., 1996).

In this review, we discuss four specific interactions between earthworms and microorganisms which are considered as hot spots of microbial and earthworm activities at a microscale: nephridial symbiosis, food preference, gut symbiosis and microorganisms in burrows and casts.

2. Nephridial symbiosis

Some apparently species-specific microbial symbionts in the ampullas of the nephridia have been described on the basis of microscopic observations in earthworm species (Knop, 1926). Nephridia are paired osmoregulatory-excretory organs present in each segment of the earthworm body (Laverack, 1963). The presence of symbionts in nephridia was confirmed as being members of a monophyletic branch of the genus *Acidovorax* (Schramm et al., 2003). Their results show that different species of earthworms harbour distinct gene sequence types of the associated *Acidovorax* sp. whereas the same earthworm species from different continents present similar symbiont sequences. These bacteria might be proteolytically active during excretion, facilitating the absorption of peptides and amino acids by hosts (Pandazis, 1931). To what extent, this association influences nitrogen excretion by earthworms and thus the nitrogen cycle in soil is still unknown (Schramm et al., 2003). Since the discovery of symbionts (Acidovorax-like bacteria) in *E. fetida* nephridia, several studies regarding bacterial colonization of earthworm nephridia have been reported (Davidson et al., 2006; Davidson et al., 2008; Pinel et al., 2008; Davidson et al., 2010; Lund et al., 2010). Firstly, Davidson et al. (2006) demonstrated that these nephridial symbionts are directly transferred from the adults to the egg capsules during mating and are not acquired from the environment in *E. fetida*. *Acidovorax* cells, present in nephridia are dominant in mating mucus and in egg capsules. A subsequent study shows that the model for colonisation of the nephridia embryo is a sequential acquisition that begins when a nephridial canal matures in a segment and releases an attractant, inducing selective migration of the *Acidovorax*-like bacteria (Davidson et al., 2008). Gene sequence phylogenies, based on 16S rRNA comparison, revealed that all earthworm symbionts formed a cohesive and independent group (Pinel et al., 2008). After the isolation and the characterization of a relative of the genus *Acidovorax* from nephridia of *E.*

fetida and based on the unique ecology of this organism, a new genus and a new specie, respectively *Verminephrobacter* and *Verminephrobacter eiseniae,* have been defined (Pinel et al., 2008). The presence of symbiotic bacteria of the genus *Verminephrobacter* in several earthworm species was then investigated (Lund et al., 2010). This study shows that of over 23 earthworm species, 19 have *Verminephrobacter* symbionts in their nephridia. The studied species can be divided into three categories:

- Species with nephridia only colonised by *Verminephrobacter* symbionts, including *Lumbricus terrestris* L., *Aporrectodea caliginosa* Savigny, *Aporrectodea longa* Ude
- Species with nephridia colonised by a mixed bacterial population with *Verminephrobacter* symbionts, including *Aporrectodea rosea* Savigny, *Dendrobaena veneta* Bouché.
- Species with nephridia free of *Verminephrobacter* symbionts, including *Dendrobaena octaedra* Savigny and *Dendrobaena attemsi* Michaelsen.

New evidence reveals a more complex symbiosis system in *E. fetida* nephridia, involving three bacterial symbionts from different classes: *V. eiseniae*, a *Microbacteriaceae* and a *Flexibacteriaceae*. The presence of these three bacteria in the egg capsule and adults confirmed that they are associated symbionts of *E. fetida* and transmitted to the next generation (Davidson et al., 2010).

The role of these bacteria in capsule eggs remains uncertain but due to the diversity of microbial pathogen and potential predators in the soil community, a role of chemical protection is plausible (Davidson et al., 2010).

3. Food preference

Microorganisms are an unavoidable constituent of earthworms' natural diet (Edwards et al., 1996). Some microbes are preferentially ingested by earthworms while others are rejected. Previous studies have highlighted selective feeding strategies in various earthworm species for certain fungal and bacterial species (Satchell, 1967; Doube et al., 1997; Doube et al., 1998; Neilson et al., 2003). In multiple choice tests, *L. terrestris* preferred apple leaves and paper discs inoculated with microorganisms and showed distinct preference for two soil fungi, *Mucor hiemalis* Wehmer and *Penicillium* sp. rather than a bacterium, *Pseudomonas fluorescens* Migula, indicating that fungal growth on food substrates may enhance the availability of carbohydrates and nitrogenous compounds to earthworms (Wright, 1972; Cooke et al., 1980). Bonkowski et al. (2000) carried out selection experiences in order to study preference of earthworm species for a variety of soil fungi. Nine fungal species were

proposed to five different earthworm species. They found that two fungal species, *Fusarium nivale* Müll and *Cladosporium cladosporioides* de Vries, were preferred by earthworms. They concluded that earthworms used early successional fungal species as cues to detect fresh organic food sources in soil but the nature of this preference is unknown. Food preference tests conducted with *D. octaedra*, *A. caliginosa* and *Octolasion tyrtaeum* Savigny show that these earthworms preferred organic matter inoculated with different actinomycete species in comparison with a control (organic matter without actinomycetes) (Jayasinghe et al., 2009). This is the first example of earthworm food preference for actinomycetes. How earthworms forage and select for food resources remain unsolved. The evidence of the presence chemoreceptors in earthworms (Laverack, 1960) combined with their ability to select specific food materials, has led to the hypothesis that olfaction could be used by earthworms in their search for adequate nutriments including microorganisms. Indeed, most living organisms have developed sensory modalities based on principles of neural organisation in order to detect and react to chemicals present in their external environment (Hildebrand, 1995). Moreover, semiochemical compounds govern intra- and inter-specific communication in a great diversity of organisms (Wyatt, 2003) including plants (Runyon et al., 2006) and insects (De Moraes et al., 2001), and are frequently used in agricultural applications (i.e. mating disruption, pheromone traps, push-pull strategies) (Hardie et al., 1999; Verheggen et al., 2010). Further experiments seem necessary to develop this hypothesis and enhance our knowledge on earthworm food preference.

4. Gut symbiosis

Some microorganism species were submitted to growing stimulation during gut transit (Edwards et al., 1996). Indeed, the survival of microorganisms in the earthworm gut depends on their capacity to resist digestive enzymes of microbial or earthworm origins, intestinal mucus, $CaCO_3$, or to bacteriostatic and microbial substances (Brown, 1995) and also transit time (Scheu, 1992).

Analysis of the digestive tract contents in earthworms has revealed the presence of grass fragments and other plant leaves, roots, algal cells, seeds, fungi, bacteria, protozoa, and actinomycetes (Piearce, 1978). The microbial composition of earthworm intestine contents has been considered to reflect that of the soil or ingested plant remains (Morgan, 1988; Brown, 1995; Brown et al., 2004), but there is evidence of the possible existence of ecological group-specific gut microbiota in some earthworm species (Lavelle et al., 2001). Indeed, some physical links were found between bacterial cells and epithelium in the hindgut of *L. terrestris*

(Jolly et al., 1993). The presence of a mutualistic digestive system was demonstrated in several tropical and temperate earthworm species in which soluble organic carbon, in the form of a mixture of low-molecular weight mucus, was added to enhance the soil microflora proliferation (Lavelle et al., 1995; Trigo et al., 1999). Intestinal mucus is composed of amino acids (about 200 Da) mixed with high-molecular weight sugars and glycoproteins (40000-60000 Da) (Martin et al., 1987). The mucus production and the enzyme pool depend on earthworm species and food quality. For example, epigeic species, which feed on rich substrates, need a complex enzymatic system but not an intensive mucus production in their gut (Trigo et al., 1999). More recent research has focused on the composition and structure of microbial populations of the earthworm's digestive tract (Furlong et al., 2002; Schönholzer et al., 2002; Horn et al., 2003; Ihssen et al., 2003). Furlong et al. (2002) have shown that some microorganisms of soil (e.g., *Pseudomonas* sp. and *Firmicutes* sp.) increase in abundance through the gut tract of *L. rubellus*. Automated image analysis and in situ hybridization were used to study the gut transit impact on bacterial community structure (Schönholzer et al., 2002). Moreover, earthworm gut was identified as an ideal habitat for N_2O-producing bacteria because earthworms activate these microorganisms during gut passage (Horn et al., 2003; Ihssen et al., 2003).However, a comprehensive description of the digestive system and the origin of different gut enzymes require further research, particularly for epigeic and anecic species (Brown et al., 2000). An increasing appreciation of the synergistic interactions between earthworms and microorganisms is observed. The main interest is focused on microorganisms that are ingested from soil and transit the gut by employing culture-based and molecular methods (Egert et al., 2004; Parthasarathi et al., 2007; Byzov et al., 2009; Thakuria et al., 2009). Despite those recent studies, the real existence of symbionts in the earthworm gut is still controversial (Curry et al., 2007). Some show that microbial fingerprints in the earthworm gut are associated to the microbial profile in soil and in food sources (McLean et al., 2006; Drake et al., 2007; Knapp et al., 2008; Jayasinghe et al., 2009). For instance, only small differences in bacterial communities between soil, gut, and fresh casts of *L. terrestris* have been highlighted, suggesting that the existence of an indigenous earthworm microbial community is unlikely (Egert et al., 2004). Similar results were found by Knapp et al. (2009) during their study on the impact of a radical diet shift on gut microbiota of *Lumbricus rubellus* Hoffmeister and by Jayasinghe et al. (2009) where all the actinomycetes isolated from the casts occurred in the natural soil of their study. However, other studies show some evidence of earthworm gut symbionts (Sampedro et al., 2007; Byzov et al., 2009). They found some microorganisms in the earthworm intestine that are absent in the surrounding soil

(Byzov et al., 2009) and important changes in the fatty acid concentration and composition in the gut of the earthworm *L. terrestris* (Sampedro et al., 2007). Moreover, a recent study showed that the development of distinct gut wall-associated bacterial communities is strongly associated to earthworm ecological group despite the shift observed with food source and habitat changes. Presence of all bacteria in earthworm gut and in soil does not allow determination of whether the bacterial communities share a symbiotic or a mutualistic metabolic interaction with earthworms (Thakuria et al., 2010).

Further studies in a large diversity of earthworm species are necessary to confirm the real presence of symbionts in earthworm gut and their functional role.

5. Microorganisms in burrow and casts

For some microorganisms, earthworms' gut represent mobile micro-habitats in which dormant microorganisms can find available food, mobility and shelter to develop (Lavelle et al., 1995). The activities of these microorganisms can continue for a short time in the casts because of the suitable amount of soluble carbon and nutrient resources. For example, an increase of microbial respiration rate (approximately 90%) is observed in fresh cast of *A. caliginosa* (Scheu, 1987). As in earthworm gut and casts, a concentration effect of microorganisms and invertebrate activity has been observed in burrows (Graff, 1971; Brown, 1995; Tiunov et al., 1999; Jégou et al., 2001). *Cellulomonas* sp. and *Promicromonospora* sp. were the dominant bacteria in *L. terrestris* burrow walls, whereas *Bacillus* sp. and *Streptomyces* sp. prevailed in the surrounding soil. But no specific fungal community was observed in the burrows of *Lumbricus terrestris* (Tiunov et al., 2002). The presence of microorganisms in earthworm burrows can probably be explained by the loss of carbon through the mucus secretion of earthworms and nitrogen secretion through nephridia (Brown et al., 2000). Recent studies confirm a more intensive microbial activity in earthworm burrow and cast (Amador et al., 2007; Jayasinghe et al., 2009). For different culture media, the number of cells/g is higher in structures built by earthworms including burrows and casts than bulk soil (Amador et al., 2007). Moreover, actinomycetes counts in casts of three earthworm species are significantly higher than in the natural soil, indicating that the casts may act as excellent microhabitats for the growth of these microorganisms (Jayasinghe et al., 2009).

6. Conclusions

This review was intended to summarise the present knowledge of microscale interactions between earthworms and microorganisms in soil. Relations between earthworms and

microorganisms are diverse and complex. Earthworms have symbionts in nephridia and some investigations tend to demonstrate symbiosis in earthworms guts, but future experiments are necessary in different species to generalise this association. Interactions between earthworms and microorganisms are also observed in earthworm casts and burrows, suggesting that these media increase microorganism activities. Moreover, food preferences are revealed for some earthworm species, indicating specific associations. However no study explains how earthworms can choose a particular microorganism. Chemical communication through microbial volatiles in order to identify potential biologically active compounds for earthworms could be an interesting way to explore this.

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3.3. Earthworms use odor cues to locate and feed on microorganisms in soil

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Reference: Lara Zirbes, Mark Mescher, Véronique Vrancken, Jean-Paul Wathelet, François J. Verheggen, Philippe Thonart, Eric Haubruge (2011) Earthworms use odor cues to locate and feed on microorganisms in soil. Plos One 6(7): e21927.

Abstract - Earthworms are key components of temperate soil ecosystems but key aspects of their ecology remain unexamined. Here we elucidate the role of olfactory cues in earthworm attraction to food sources and document specific chemical cues that attract *Eisenia fetida* to the soil fungi *Geotrichum candidum.* Fungi and other microorganisms are major sources of volatile emissions in soil ecosystems as well as primary food sources for earthworms, suggesting the likelihood that earthworms might profitably use olfactory cues to guide foraging behavior. Moreover, previous studies have documented earthworm movement toward microbial food sources. But, the specific olfactory cues responsible for earthworm attraction have not previously been identified. Using olfactometer assays combined with chemical analyses (GC-MS), we documented the attraction of *E. fetida* individuals to filtrate derived from *G. candidum* colonies and to two individual compounds tested in isolation: ethyl pentanoate and ethyl hexanoate. Attraction at a distance was observed when barriers prevented the worms from reaching the target stimuli, confirming the role of volatile cues. These findings enhance our understanding of the mechanisms underlying key trophic interactions in soil ecosystems and have potential implications for the extraction and collection of earthworms in vermiculture and other applied activities.

1. Introduction

Olfaction is a key sensory modality by which animals, and many other organisms, acquire information about the surrounding world [1,2]. In addition to perceiving semiochemicals (i.e., pheromones and kairomones), which play key roles in many interactions within and between species [3], many organisms also detect and respond to general odorant cues deriving from biotic and abiotic features of their environments. Indeed, most organisms have specialized sensory, information processing, and behavioral mechanisms dedicated to detecting and reacting to chemicals present in the external environment [4,5]. Among terrestrial invertebrates, the perception and use of olfactory cues by insects, and some other arthropods, has been extensively studied [2,6,7,8] as has the ecological role of olfactory cues in interactions among insects and plants (e.g.,[9,10,11,12]) Previous work has also explored the detection of olfactory cues by nematodes which entails the activation of papilla and setae on the body surface that are connected to chemosensory neurons known as the AWA cells [1,13] and the response of nematodes to various olfactory cues [14,15,16]. In contrast, olfaction by annelids remains poorly studied. Chemoreceptors have been identified on the prostonium and the buccal epithelium of earthworms [17] and have been shown to detect sucrose, glucose and quinine [18]. Recently, olfaction by earthworms has been suggested to be involved in the coordination of collective movement [19].

Previous studies exploring the feeding strategies of various earthworm species suggest that these animals exhibit orientation and movement toward particular food sources, including specific species of protozoa, bacteria, fungi and plants [20,21,22,23]. Microorganisms are both major components of earthworm diets [20] and principle sources of volatile organic compound emissions in soil ecosystems [24], suggesting that olfaction may play a key role in earthworm foraging. Moreover, Bonkowski and Schaefer [25] reported that *Aporrectodae caliginosa* actively moved toward foraging sites exhibiting higher densities of protozoa and naked amobae. Soil fungi are particularly important food sources for earthworms, especially for epigeic species that consume litter typically colonized by fungi [26,27], including *Geotrichum candidum*, *Mucor* sp., and *Aspergillus flavus* [28,29]. Bonkowski et al. [28] conducted feeding choice assays to document the preferences of five earthworm species for a variety of soil fungi and reported a general pattern in which worms exhibited a preference for early successional species (e.g., *Fusarium nivale* and *Cladosporium cladosporioides*) that are presumably indicative of relatively new and nutrient rich organic resources. The factors underlying the observed preferences were not determined, though the investigators postulated

that differences in the nutritional value of the fungi, or the presence of antibiotic compounds or other metabolites in or around the mycelia might be important.

Because soil fungi release volatile and non-volatile chemicals, as well as influencing the release of plant-derived compounds [30,31], olfactory cues associated with the presence of fungi may be expected to play an important role in earthworm foraging for fungal food sources. However, previous studies have not explicitly addressed the role of olfaction or documented the specific cues responsible for orientation and attraction. Therefore, we explored the role of olfaction in the foraging of *E. fetida*, an epigeic earthworm species with economic significance for various industrial processes, on the soil fungus *G. candidum*, which is an important food source for this worm [29].

2. Methods

2.1 *Eisenia fetida* rearing

Earthworms (*Eisenia fetida*) provided by Ouroboros s.a. (Belgium) were reared in PVC boxes (42 cm long x 30 cm wide x10 cm high) filled with universal compost DCM ® (De Ceuster Meststoffen s.a.,Grobbendonk, Belgium) composed of a mixture of brown peat, white peat, and lava. The compost was changed every two months and cocoons and hatchling earthworms were placed in new boxes with fresh compost. Boxes were maintained at 23 ± 1 °C. Only mature earthworms (with a clitellum) were used for our experiments.

2.2. Culture of *Geotrichum candidum*

Geotrichum candidum, isolated from compost mixed with milky fermented product, was cultured in 100 ml of liquid medium 863 (2 g glucose; 1 g yeast extract; 1 g peptone) at 27 ± 3°C during 42 h, and the culture was filtered with Pall Supor® (Whatman Ltd, England) - 450 membrane 47 mm – 0.45 µm filter.

2.3. Bioassays

a. Earthworm response to cues associated with *Geotrichum candidum*

A PVC box (Box #1: 56 cm x 36 cm x 8 cm) was filled with moist compost (76% humidity content; which was measured by drying a 25 g sample of moist compost at 105 °C for 48 h), and 200 earthworms (100 matures and 100 immatures) were placed randomly within it. A second box (Box #2: 37 cm x 26 cm x 9 cm) was placed on top of Box 1 (Fig. 1a). Box 2 had 5 slots (30 cm long x 0.5 cm wide) in its bottom and was also filled with moist compost (prepared as above). Filtrate (275 ml) from the *G. candidum* culture was then poured evenly

across the surface of Box 2. Pairs of control boxes were similarly placed but received tap water instead of the fungal filtrate. After 120 h, the number of earthworms in each box was determined. Six repetitions were conducted with the *G. candidum* filtrate and three for the controls.

b. Four-arm olfactometer experiments

Earthworm behavior was more precisely observed in a below-ground olfactometer consisting of a central PVC chamber (20 cm x 20 cm x 10 cm) connected to four equidistantly space side arms (9 cm in diameter, 18 cm long) (Fig. 1b). For each experiment, the entire system was filled with moist compost (as above). Target stimuli (i.e., *G. candidum* filtrate or filter paper treated with individual compounds) were placed at the far end of one arm (selected randomly), while the three remaining arms acted as controls. Between repetitions, each piece of the olfactometer was cleaned with tap water and then with norvanol before being dried overnight at 70°C. For each stimulus tested, groups of earthworms varying in number/density from 5 to 160 individuals were introduced in the central chamber and allowed to make choices (the specific numbers used for each assay are listed in table 1).

Table 1 – Treatments employed in the four-arm olfactometer bioassays**.**

Experiment	Number of earthworms	Tested substance	Quantity of tested substance	Repetitions
Influence of *G. candidum*	20	*G. candidum* filtrate	25 ml	18
Influence of earthworms density	5	*G. candidum* filtrate	25 ml	18
	10	*G. candidum* filtrate	25 ml	18
	40	*G. candidum* filtrate	25 ml	18
	80	*G. candidum* filtrate	25 ml	18
	160	*G. candidum* filtrate	25 ml	18
Influence of identified volatile compounds	20	Ethyl acetate	1ml	10
	20	Ethyl propionate	1ml	10
	20	Ethyl pentanoate	1ml	10
	20	Ethyl hexanoate	1ml	10
	20	3-octanone	1ml	10
	20	2-methylbutan-1-ol	1ml	10
	20	3-methylbutan-1-ol	1ml	10
	20	2-methylpropan-1-ol	1ml	10

One day after release, the olfactometer was disassembled, the compost in each arm was placed in a separate container, and the number of earthworms was recorded. The specific stimuli assayed are presented in table 1. When testing effects of volatile cues on earthworm behavior, a circular metal screen was placed in the middle of each arm to prevent physical contact with the target. Four different quantities of ethyl pentanoate and ethyl hexanoate (1 µl,

10 µl, 100 µl and 1000 µl) were evaluated (sample purity was 97% and 98%, respectively for the two esters). At least 10 replicates were conducted for each trial. A control with only compost in the four arms of the olfactometer was also employed in 3 replicates.

c. Vertical olfactometer experiments

A second, vertical olfactometer (Fig. 1c) was also employed to study earthworm responses to chemical cues over greater distances and to test the feasibility of using chemical cues to attract earthworms to the soil surface, as in vermicomposting. This olfactometer constituted PVC tubes 9 cm in diameter and either 25 cm, 40 cm or 105 cm long that were filled with moist compost (as above). Ten earthworms were placed at the bottom of the olfactometer and 25 ml of *G. candidum* culture filtrate was introduced at the top. After 24 h (for olfactometers having heights of 25 and 40 cm) and 65 h (for the olfactometer having a height of 105 cm), earthworms present in the top five centimeters of the olfactometer were counted. As a control, similar trials were conducted without filtrate for each olfactometer length. Each experiment was replicated 18 times.

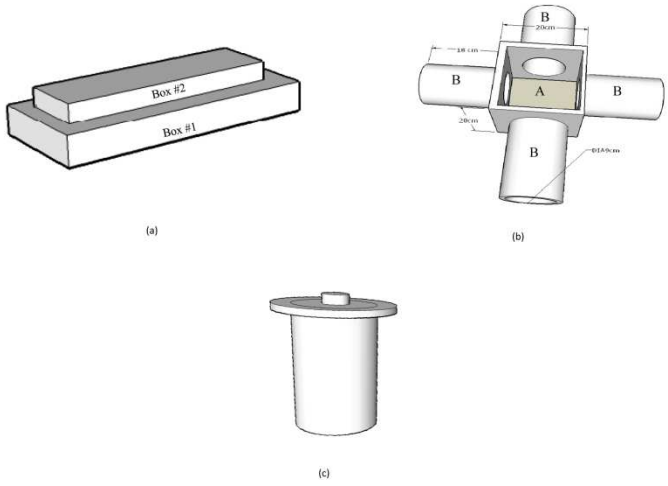

Fig. 1: Experimental set-ups. (a) Dual-box earthworm sampling device, (b) four-arm olfactometer (A = central chamber, B = (identical) arms), (c) vertical olfactometer

2.4. Sampling fungal volatiles

A 5 ml sample of *G. candidum* filtrate was placed in a glass vial with a septum (opening) in the lid. A 75 µm carboxen-polydimethylsiloxane (CAR/PDMS) solid-phase micro-extraction (SPME) (Supelco) fiber was inserted through the septum and exposed for 30 min at 40 °C. The compounds adsorbed on the fiber were analyzed by gas chromatography-mass

spectrometry (GC-MS). The GC–MS system comprised a GC (5890 Serie II Plus, Hewlett Packard) linked to a quadrupole type mass selective detector (5989A, Hewlett Packard). The fiber was inserted manually into the injector port (240°C), desorbed, and the sample chromatographed on an apolar column (Factor four VF-5ms, 30 m, 0.25 mm internal diameter, 0.25 µm film thickness, Varian). Helium at a constant pressure of 55 kPa was used as carrier gas flow. After fiber insertion, the column temperature started at 40 °C during 30 sec, increased to 180 °C at 5 °C/min then to 240 °C at 15 °C/min followed by a final hold of 2 min at 240 °C. Electron impact mass spectra were recorded over the range 30 – 350 *m*/*z* (Electron energy: 70eV). Identifications were performed by Wiley 275 library searches and by comparison with the retention time of external standards. Three replicate samples were analyzed. Volatiles of filtrated culture medium (medium 863) were collected and analyzed as controls.

2.5. Statistical analyses

A Chi-square Goodness-of-fit test (Minitab® v15.0, State College, Pennsylvania USA - α = 5%, 3 degree of freedom) was used to compare earthworms' distribution in each arm of the four-arm olfactometer to a theoretical distribution based on random preferences for each of the olfactometer arms. A one-way ANOVA (Minitab® v15.0, State College, Pennsylvania USA - α = 5%) was used to compare the numbers of earthworms in the top five centimeters of the vertical olfactometer in presence or absence of filtrate.

3. Results

To determine whether *E. fetida* respond to olfactory cues associated with *G. candidum*, responses to fungal filtrate and specific compounds were examined in semi-natural conditions using pairs of stacked boxes. Significantly more earthworms were collected in target box when *G. candidum* filtrate was applied, 179 ± 6.26 (mean ± SD) vs 87 ± 23.31 (mean ± SD) when filtrate was absent (One-way ANOVA, $p < 0.001$). Because of the long exposure time employed in this assay (120h), it is possible that some fluid components of the filtrate, in addition to olfactory cues, may have percolated through box two and arrive in box one. Subsequent experiments, described below, more effectively test attraction to volatile cues alone. Initial experiments conducted with a four-arm olfactometer produced similar results, as significantly more earthworms were recovered from the olfactometer arms treated with *G. candidum* filtrate (Fig. 2a; Chi-square Goodness-of-fit test, $\chi^2_3 = 34.44$, $p < 0.001$).

There was no apparent bias in the experimental set-up, as a fairly uniform distribution of earthworms across the 4 olfactometer arms was observed on controls where filtrate was not introduced. (Arm1: 7; arm2: 7, arm3: 3, arm4: 5; Chi-square Goodness-of-fit test, $\chi^2_3 = 2$, $p = 0.572$). Similar earthworm attraction was observed across a range of population densities (Table 2).

Table 2 – Influence of earthworm density on earthworm attraction in the four-arm olfactometer. Total earthworm number across all replicates is given in the table.

	Earthworms density					
	5 earthworms	10 earthworms	20 earthworms	40 earthworms	80 earthworms	160 earthworms
G. candidum	49	83	92	326	528	1536
Control 1	5	19	45	64	156	202
Control 2	11	18	47	60	127	169
Control 3	3	15	36	55	126	138
χ^2	82.35	96.08	34.44	421.7	493.63	2742.69
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Moreover, in experiments conducted with three vertical olfactometers (25, 40 and 105 cm) earthworms reached the soil surface only when *G. candidum* filtrate was present (Table 3), demonstrating vertical attraction to olfactory cues over significant distances and thus the feasibility of collecting earthworms at soil surface with *G. candidum* filtrate.

Table 3 – Earthworm behavior in vertical olfactometer. Numbers of earthworms (mean ± SD) collected in the top 5 cm of the vertical olfactometer in presence and in absence of *G. candidum* filtrate for each olfactometer arm length.

	Olfactometer height		
	25cm	40cm	105cm
With *G. candidum* filtrate	8.3 ± 0.3	6.9 ± 0.2	6.1 ± 0.4
Without *G. candidum* filtrate	1.3 ± 0.3	1.3 ± 0.3	0.4 ± 0.1
p-value	< 0.001	< 0.001	< 0.001

To determine the olfactory cues responsible for the observed attraction, volatiles from *G. candidum* filtrate were collected by SPME and analyzed by GC-MS. These analyses identified 18 molecules, of which 16 were specifically identified from filtrate of *G. candidum*: ethyl acetate, 2-methyl-1-propanol, ethyl propionate, 3-methyl-1-butanol, 2-methyl-1-butanol, ethyl 2-methylpropanoate, ethyl butanoate, ethyl but-2-enoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl pentanoate, ethyl 3-methylbut-2-enoate, ethyl 2-methylbut-2-enoate, 3-octanone, ethyl hexanoate, ethyl hex-2-enoate. Authentic standards of eight molecules that

were commercially available were tested separately in the four-arm olfactometer. Two esters exhibited significant attraction of *E. fetida*: ethyl pentanoate (Chi-square Goodness-of-fit test, $\chi^2_3 = 0.3105$, $p < 0.001$) and ethyl hexanoate (Chi-square Goodness-of-fit test, $\chi^2_3 = 0.2173$, $p < 0.001$) (Fig. 2b and 2c).

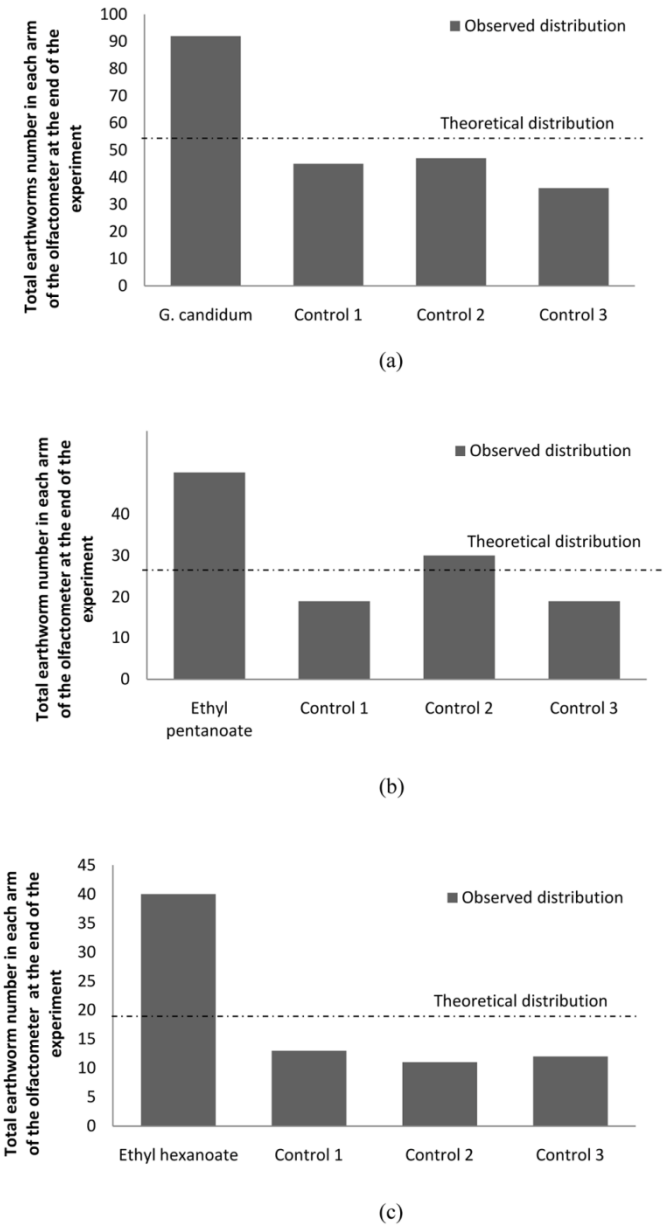

Fig. 2: Earthworm behavior in four arm olfactometer. Observed and theoretical (i.e. random) distributions of earthworms in each arm of the four-arm olfactometer when one arm is treated with (a) *G. candidum* filtrate, (b) ethyl pentanoate (100% v/v), or (c) ethyl hexanoate (100% v/v). The distributions are compared by a Chi-square Goodness-of-fit test

The poor water solubility of these molecules suggests that volatile cues, diffusing in compost, are likely attractants.

To confirm that volatile cues were responsible for attraction, attraction to the two esters was measured in the four-arm olfactometer using a metallic mesh to prevent the earthworms from contacting the odor source (thus ruling out contact cues). Significant attraction was observed for ethyl pentanoate at quantities above 10µl and for ethyl hexanoate at quantities above 100µl, and weak attraction was observed for both compounds even at levels as low as 1µl (Table 4).

Table 4 – Quantities of ethyl pentanoate and ethyl hexanoate tested and *E. fetida* responses to each. - = no attraction, ◊ = earthworm attraction. (Note: Although p-values at 1µl for both esters are significant (and in each case, earthworms were overrepresented in the treatment arm relative to expectations based on a random distribution) attraction to the treatment arm was not significantly different than to at least one adjacent control arm. Instead earthworms were significantly underrepresented in the most distant control arm. This result is consistent with weak attraction to this low concentration of the target compound.

Molecules	Quantity	Attraction	p-value
Ethyl pentanoate	1µl	◊	0.032
	10µl	◊	0.006
	100µl	◊	< 0.001
	1000µl	◊	< 0.001
Ethyl hexanoate	1µl	◊	0.030
	10µl	-	0.67
	100µl	◊	< 0.001
	1000µl	◊	< 0.001

4. Discussion

Our results clearly demonstrate that *E. fetida* are attracted by olfactory cues associated with *G candidum*, and thus complement previous reports that earthworms are able to actively search for food sources [20]. We furthermore identified two specific compounds from the filtrate of *G. candidum* colonies that exhibit significant attraction for *E. fetida*, the esters ethyl pentanoate and ethyl hexanoate. No previous studies have identified specific olfactory cues used by earthworms. In nematodes, attraction has been shown for unidentified olfactory cues deriving from insect larvae [32], and for several specific chemical compounds, including diacetyl, (E)-ß-caryophyllene, isobutanol [14,15]. In *C. elegans*, chemiotaxis to volatiles were observed for at least 50 compounds, and specific neurons and genes involved for these responses have been described [1,33]. The perception of volatile odorants by *E. fetida* may also involve some specific corporal receptors associated with neurons. Indeed, earthworms are known to have chemoreceptors, principally on the prostonium or on the buccal epithelium that are associated with the nervous system and more particularly with axons and dendrites [18]. The foraging strategy of *E. fetida* may also bear similarity to social strains of *C. elegans*, as

these worms have been observed to aggregate in areas where bacteria are numerous [34] and there is some evidence for coordinated movement in *E. fetida* [19].

The two esters we found to be attractive to *E. fetida* have previously been shown to function as cues for insects in other systems. Ethyl pentanoate has an attractant activity for the dung beetle, *Pachylomerus femoralis* [35], and ethyl hexanoate, in combination with 1,8-cineole and hexanol, attracts the Mexican fruit fly, *Anastrepha ludens*, to fermenting, immature fruit of yellow chapote [36]. The latter compound also stimulates upwind flight of the lepidopteran, *Ectomyelois ceratoniae* [37].

Among the molecules we identified from *G. candidum* filtrate, ethyl propionate, ethyl acetate, 3-methylbutan-1-ol, 2-methylbutan-1-ol and 2-methylpropanol have previously been found in the volatile profile of *G. candidum* and other microorganisms [38,39]. The formation of 2-methylpropanol, 2-methylbutanol, and 3-methylbutanol by *G. candidum* almost certainly involves deamination of glutamic and aspartic acids and of leucine, phenylalanine and methionine, which are commonly found in fungi [40,41]. Two other molecules emitted by *G. candidum* filtrate, hexanoic acid ethyl ester and 3-octanone, were previously identified as volatiles from the fungi *Aspergillus candidus* [39]. Different strains of lactic acid bacteria are able to synthesize ethyl ester from 2 to 10 carbon atoms, mainly ethyl hexanoate [42]. The hydrolysis products of *G. candidum* lipases may be the precursors of various volatile compounds such as alcohols, methyl ketones and esters [40].

Earthworm attraction to chemical cues associated with food has potential application for the development of techniques for the extraction and sampling of earthworms, for example in vermicomposting. Other behavioral techniques have previously been employed for such purposes, including heat extraction, electrical extraction, and mechanical vibration [18,43,44], and chemical extraction methods using natural repellents or irritants, like formalin, mustard extract, exotic-plant extracts have been reported [45,46,47,48,49]. Because they are based on attraction rather than repulsion, the esters presented above may have advantages over the existing chemical methods (e.g., efficacy when applied at low concentrations and on restricted spatial scales) but confirming this will require further study.

In conclusion, this study provides the first documentation of specific olfactory cues involved in annelid foraging. Microbiota are key producers of volatile compounds in soil ecosystems [24] as well as major components of earthworm diets [20]. Thus, further elucidation of the mechanisms by which earthworms perceive and respond to olfactory cues will enhance our understanding of the ecology of soil ecosystems, in which earthworms play a tremendously important role in temperate regions. Furthermore, exploration of earthworm olfaction will

help us to understand how these animals orient themselves and coordinate their behavior. For example, it has previously been suggested that chemical cues are involved in earthworms [18,50], and there is some evidence that *L. terrestris* follows mucus trails to find its partner [51], but the role of volatile perception in such interactions remains to be documented. Finally, as noted above, such work has potential implications for the development of techniques for the extraction and sampling of earthworms in vermicomposting and other applied settings.

5. Acknowledgements

We thank the members of the group of E. Haubruge for their continuous support; D. Conoir for his technical assistance; and Ouroboros s.a. society for providing *E. fetida* earthworms.

6. Author Contributions

Conceived and designed the experiments: LZ EH. Performed the experiments: LZ VV. Analyzed the data: LZ. Contributed reagents/materials/analysis tools: EH J-PW PT. Wrote the paper: LZ FV MM.

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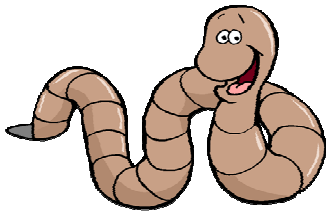
Chapter 4: Intra-specific interactions in Eisenia fetida (Savigny, 1826)



4.1. General introduction

As previously mentioned, semiochemical compounds govern intra- and inter-specific communication in a great diversity of organisms including plants, insects and nematodes, and are frequently used in agricultural applications (e.g. mating disruption, pheromone traps and push-pull strategies). More pertinently, semiochemical compounds are implicated in intra-specific aggregation and mass migration, for example in cockroaches, locusts, aquatic whirligig beetles, and tribolium. Intra-specific interactions have been investigated in 15 lumbricid species. These interactions vary strongly with population density, and for *Eisenia fetida*, density dependence is suggested to be the key mechanisms regulating population dynamics. Moreover, modifications in intra-specific interactions by chemical compounds in *E. fetida* and *E. andrei* have been suggested.

The role of volatile compounds in the food foraging behaviour of earthworms has been demonstrated in Chapter 3 but no existing literature describes intra-specific interaction in earthworms and any potential related role of volatile compounds. During several experiments, earthworms groups and unexpected self-assembled *Eisenia fetida* balls have been observed out of soil or in rearing substrates. Many preliminary observations strongly suggest that such structures require necessarily intra-specific interactions and self-assemblage. Moreover, collective movement is sometimes observed in earthworm populations; groups evade unfavourable environmental conditions like flooded soil and low oxygen tension, or seek new habitats. Occasionally, the number of earthworms migrating is especially significant, suggesting that social cues may be responsible for collective movement. Chemical and/or physical ‘communication’ could occur in earthworms. The aim of this chapter was to study role of volatile compounds in cluster formation and collective movement of *Eisenia fetida*.

4.2. Self-assemblage and quorum in *Eisenia fetida* (Oligochaete, Lumbricidae)

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Reference: Lara Zirbes, Yves Brostaux, Mark Mescher, Maxime Jason, Eric Haubruge, Jean-Louis Deneubourg (2011) Self-assemblage and quorum in *Eisenia fetida* (Oligochaete, Lumbricidae). Plos One (Under review)

Summary - Earthworms are key components of temperate soil ecosystems but specific aspects of their behaviour remain unexamined. While social aggregation is largely studied in many biological systems, very few studies deal with intra-specific interactions in earthworms and nothing is known about cluster formation. Using circular arena and 2-arm olfactometer assays combined with mathematical modelling, we documented the *Eisenia fetida* (an economically important species of earthworms, especially in waste management) ability to join and leave a cluster. This study identifies, for the first time, self-assembled structures in earthworms and clarifies the mechanisms of cluster formation for the compost earthworm: the probability of joining a group increased with the group size of earthworms whereas the probability of leaving it decreased. Attraction at a distance was observed, suggesting the role of volatile cues in cluster formation. The cluster survival presents an all-or-one phenomenon or quorum, confirming the importance of a great number of earthworms in the cluster for its stability. These findings enhance our understanding of intra-specific interactions in earthworms and have potential implications for extraction and collection of earthworms in vermicomposting processes

1. Introduction

Spatial distribution of individuals determines the level of interaction between individuals, the structure and the organization of population [1]. Aggregation is reported for many species from microorganisms to vertebrates [2-5]. The spatial heterogeneity of the aggregated population depends, on its environment including food distribution, habitat fitness, light and temperature gradients in addition to other abiotic parameters [6]. The final aggregate are formed by individuals responding to the same environmental stimuli without social relationships [7]. Alternatively, spatial heterogeneity depends on mutual attraction between individuals in a group [4]. In these social cases, groups find their origin and their cohesion in the inter-attraction between individuals [3,7-9]. The dynamics of aggregation survival depends on the probability of individuals to leave an aggregate and/or the probability of joining it. The population size influences these probabilities (e.g. [8,10,11]). The self-assemblage, as described in insect societies [12,13], is a particular structure in social aggregates. Self-assemblage is defined as a "physical structure comprised of individuals that have linked themselves to one another" [12]. The functions of self-assemblage formations were grouped under five broad categories by Anderson et al. [12]: defence, pulling structures, thermoregulation, colony survival under inclement conditions and ease of passage when crossing an obstacle. These functions are not mutually exclusive. Detailed information on mechanisms involved in group formation and more particularly in self-assemblage would lead to a better understanding of their ecological functions [14]. But this subject is widely understudied and earthworms are totally neglected. In *C. elegans*, genes implicated in aggregation behaviour have been identified [15] but the mechanisms of group formation remain unsolved. In earthworms, intra-specific interactions have been shown [16,17] and collective movement in *Eisenia fetida* (Savigny, 1826) have been recently studied [18].

E. fetida belongs to the epigeic earthworms group [19]. This ecological category lives on or near the soil surface, typically in the litter layers of forest soils or organic rich materials (such as compost) and does not burrow [20-22]. Epigeics feed on litter and/or the attached micro-flora and ingest little mineral soils [21].

Patchy distribution in annelids [23] and more particularly in lumbricids including *E. fetida* has been previously identified [17,24,25]. Surprisingly, we have observed self-assembled *E. fetida* clusters out of soil or in rearing substrates (Figures 1a and 1b).

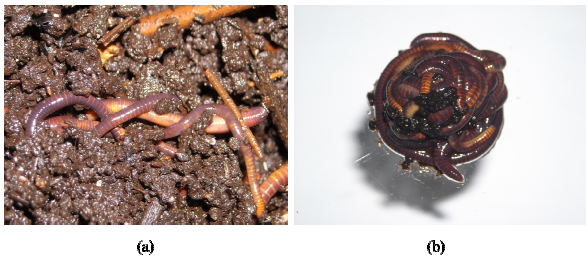


Figure 1 – Earthworms clusters. (a) Earthworms group in rearing box and (b) self-assembled earthworm cluster formed out of soil.

Substrate heterogeneities could be at the origin of these earthworm groups. Several authors have studied the distribution of earthworm communities and the influence of inter-specific relationships, soil physical and chemical factors [20,26]. However, many observations strongly suggest that such structure necessarily requires intra-specific interactions and self-assemblage [27,28].

The present work aims were to clarify the mechanisms governing cluster formation (probability of leaving and joining a group). Experimental results and a theoretical model with few functioning rules were used to identify and quantify the mechanisms of aggregation which were density dependent

2. Methods

2.1. *Eisenia fetida* rearing

Earthworms (*Eisenia fetida*) were provided by Ouroboros s.a. (Belgium) and were reared in PVC boxes (42 cm long x 30 cm wide x 10 cm high) filled with universal compost DCM ® (De Ceuster Meststoffen s.a.,Grobbendonk, Belgium). The compost was changed every two months and cocoons and hatchling earthworms were placed in new boxes with fresh compost. Boxes were maintained at 23 ± 1 °C. Only mature earthworms (with a clitellum) were used in following experiments.

2.2. Experimental procedure

The ambient temperature for all experiments was 20 ± 1°C and ambient relative humidity was 62.5%. Experiments were realised under red light in order not to disturb the earthworms [21].

a. Assays on the probability of joining a group

To determine whether chemical compounds emitted by *E. fetida* attract conspecifics, a Y binary choice set-up was used. The set-up consisted of a Y binary choice device with 2

identical circle chambers (diameter: 3.5cm) (Figure 2a). The neutral area was 2.5cm long, three distances were tested to symmetric lateral branches of Y device (2, 2.5 and 3cm). A tissue mesh was placed at the junction between circle chambers and Y lateral branches to avoid earthworms from the group coming into the Y set-up. 15 adults of *E. fetida* were taken from the rearing box, washed with tap water, and placed randomly in one of the circle chambers. One earthworm was placed at the starting point of the set-up and earthworm direction was recorded using a numerical camera JVC® (Everio GZ-MG333) during a 45-min period. The time to make its choice was also determined. The earthworm was considered to have made a choice when it touched the tissue mesh junction. For the three lengths (2, 2.5 and 3cm), 30 repetitions were realised. Thirty similar experiments for 3cm with no earthworms in the circle were realised as a control. The set-up was washed with norvanol between each experiment.

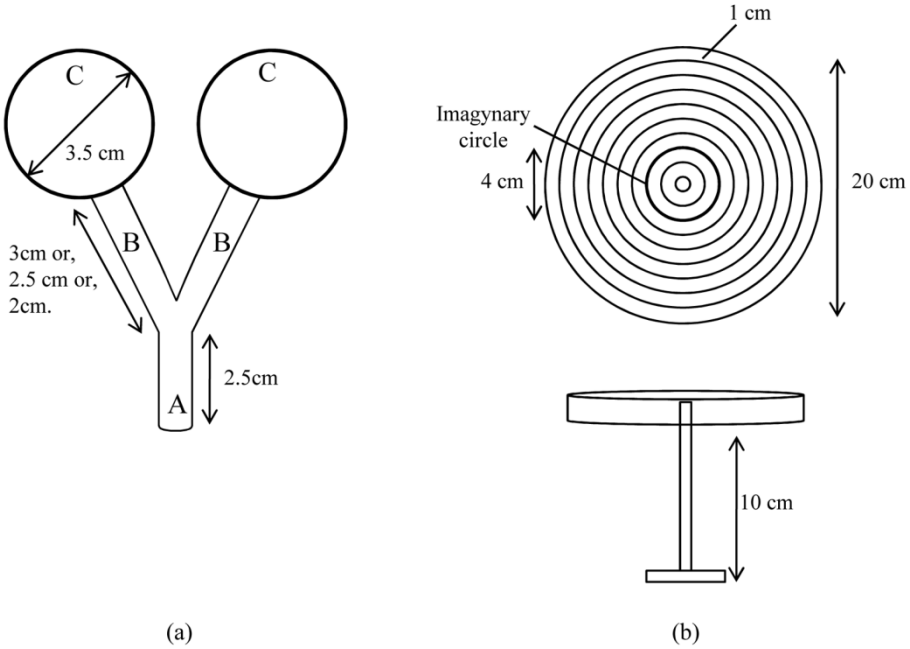


Figure 2 – Y binary choice and arena set-ups. (a) Y device. A = neutral area of 2.5 cm long where a single earthworm is placed at the beginning of the experiments, B = two lateral branches (3, 2.5 or 2cm long), C = Circle chambers of 3.5cm diam. where 15 or 0 earthworms were placed during the experiments, (b) Lateral and frontal view of the circular glass arena of 20 cm diam. with transparent sheet with concentric circles and imaginary circle of 4 cm diam. The glass arena was raised by 10 cm

In a second step, adults of *E. fetida* (0, 10 or 15 earthworms depending on the experiment) were taken from the rearing box and washed with tap water. They were placed in the centre of the arena, in an imaginary circle (diameter: 4cm). The set-up consisted of a non delimited

circular glass arena (diameter: 20 cm). To prevent earthworms from escaping, the arena was raised by 10 cm. Under the glass, a transparent sheet, with concentric circles separated from 1cm, was fixed (Figure 2b). The set-up was washed with norvanol between each experiment. A single earthworm was placed midway between the centre and the edge (between the 6^{th} and 7^{th} concentric circle). The number of earthworms reaching the imaginary central circle (with or without earthworms) or the edge, and their time to do it were recorded using a camera during a 45-min period. Time of earthworm reaction (when earthworm started moving) was also recorded. The experiment was repeated 46 times for each cluster size.

b. Assays on the probability of leaving a group

For the experiments, adults of *E. fetida* (1, 2, 5 or 10 earthworms depending on the experiment) were taken from the rearing box and washed with tap water. They were placed at the centre of the arena (the same set-up as previously), in the imaginary circle (diameter: 4cm). From this introduction and during 90 min, the number of earthworms which left the central area and the time to leave this area were recorded using a camera. The experiment was repeated 30 times for each starting number of earthworms. The set-up was washed with norvanol between each experiment.

2.3. Data analysis

In the Y set-up, a chi-square test for independence (Minitab® v15.0 State College, Pennsylvania USA - df = 2, α = 5%) was used to study the influence of lateral branch lengths on earthworm choices and a chi-square goodness-of-fit test (Minitab® v15.0, df = 1, α = 5%) to determine earthworm attraction by conspecifics. Influence of Y lateral branches length and earthworm choices on earthworm times to choose was studied with a general linear model test with 2 factors (Minitab® v15.0, α = 5%).

In the assay on the probability of joining a group with circle arena, a chi-square test for independence (Minitab® v15.0, N = 46, df = 2, α = 5%) was used to compare the number of earthworms reaching the central area according to earthworm number in this area. A general linear model with 1 factor (Minitab® v15.0, α = 5%) was used to compare time of earthworm reaction (when earthworm started moving) and the time to reach the imaginary central circle (or the edge) according to the cluster size.

In assays on the probability of leaving a group, a one-way ANOVA test (Minitab® v15.0, α = 5%) was used to compare the first earthworm time to leave the imaginary central circle or the earthworm group (N = 30). A difference test of the empiric survival curves estimated on the

same data was also conducted to compare the kinetic of this assay (R 2.10.0, R Development Core Team, $\alpha = 5\%$). This statistical test was also realised to study the departure of the second earthworm in a group. General linear model tests were used to compare leaving time of the second earthworm according to the cluster size, to compare leaving time of the second earthworm in a group of two according to contact or not between earthworms..

3. Results

Two mechanisms to explain earthworm clusters formation were explored: the probability of joining a group and that of leaving it.

3.1. Assays on the probability of joining a group

Table 1 shows results for dual choice assays using the Y set-up. Distances (lengths of Y set-up lateral branches) did not influence earthworm choice (Independence chi-square test, $\chi^2_2=1.92$, $p = 0.382$), or earthworm time to choose one of the olfactometer arm (General linear model with 2 factors, $F_{2,62} = 0.08$, $p = 0.92$). We have therefore grouped the 90 replicates. Generally, we observed two typical behaviours. In some cases, earthworms moved directly from start point and chose rapidly a branch and in other cases, earthworms explored with neutral area and one or two branches before to make the final choice (by touching tissue mesh junction). On the 90 repetitions, 22 singled earthworms made no choice: Sometimes, they stayed immobilised in the neutral area and sometimes, they explored lateral branches without touching the tissue mesh junction. A group of 15 earthworms was able to attract an isolated earthworm (Chi-square goodness-of-fit test: $N = 68$, $\chi^2_1=11.53$, $p = 0.001$). Indeed, significantly more earthworms (70.5%) chose the direction connected with the earthworm group. The earthworm choices influenced the earthworm times to make a choice (General linear model with 2 factors, $F_{1,62} = 31.51$, $p = 0.005$). Indeed, this time was longer when earthworms were present in one of the chamber of the Y set-up, 12.9 ± 1.1 min vs. 6.7 ± 0.5 min when earthworms were absent. The results were not biased by experimental set-up: after having conducted 30 tests with no source (no earthworm aggregate as stimuli) the number of earthworm was not significantly different in both arms of the Y set-up, and half of the tested worms did not initiate movement (right = 7, left = 8, no choice = 15).

Table 1 – Earthworm choices and choice times for joining assays in Y set-up

	Experiment number				**Average time (min) + SD**			
	3cm	*2.5cm*	*2cm*	*Grouped*	*3cm*	*2.5cm*	*2cm*	*Grouped*
No choice	7	10	5	22	45	45	45	45
Group way	14	16	18	48	11.68 ± 12.7	13.18 ± 11.9	13.28 ± 13.2	12.8 ± 12.4
Other way	9	4	7	20	7.5 ± 7.3	6.75 ± 9.2	6.64 ± 3.77	7.1 ± 6.4
Total	30	30	30	90				

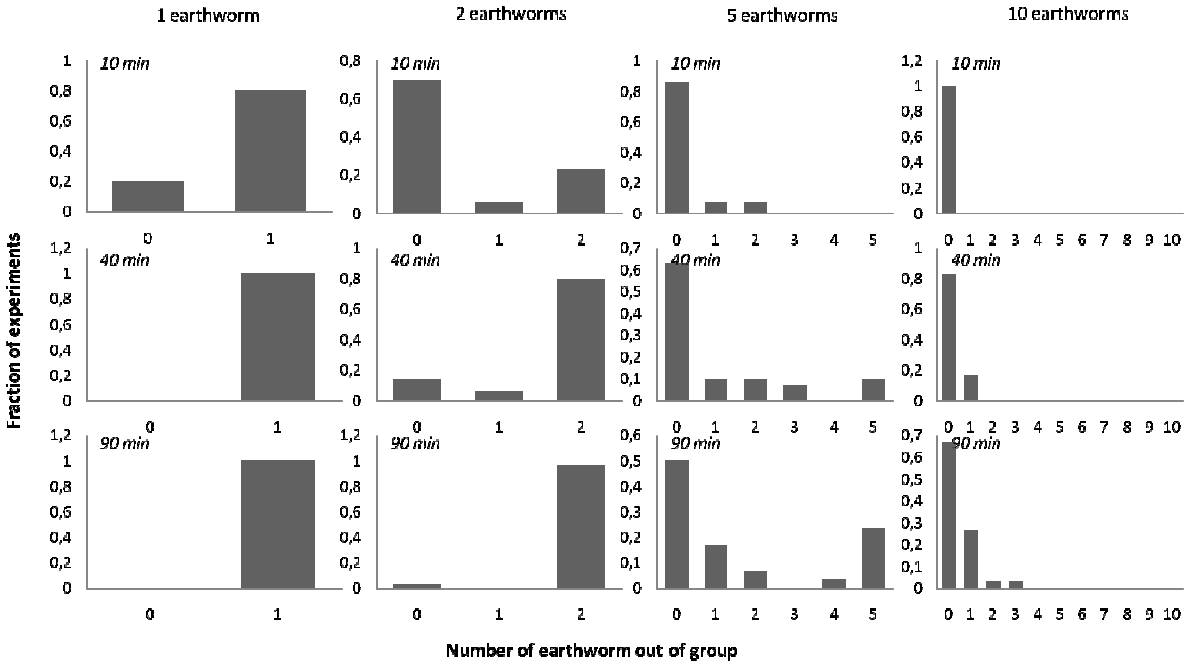
Attraction was confirmed by an experiment in a circular arena. In these experimentations, singled earthworms generally started to move only with the anterior part of their body (the head) to explore the surrounding environment. After that they moved on arena to reach the centre, or the edge. Table 2 shows the total number of isolated earthworms which reached the central area (imaginary circle in the centre of circular arena) or the edge for each cluster size, and the average times of earthworm reaction and of the meeting with the centre of the arena. In 5 repetitions, isolated earthworms did not reach the edge or the centre of the arena. In each case, earthworms wound and were immobilised during 45 min. Time to observe earthworm reaction (when earthworm started moving) (General linear model with 1 factor, $F_{2,130} = 2.15$, $p = 0.12$) and time to reach the imaginary central circle (with or without earthworm) (General linear model with 1 factor, $F_{2,43} = 0.16$ $p = 0.85$) were constant and independent of cluster size (similar results were found with earthworm time to reach the edge) whereas significantly more earthworms reached the central area of the set-up when earthworms were present (Independence chi-square test, $\chi^2_2 = 13.095$, $p = 0.001$). Moreover, the earthworm number reaching the central area was linearly dependent of cluster size ($y = 19.1x – 2.73$; $r^2 = 0.99$).

Table 2 – The results of joining assays in arena. The total number of isolated earthworms which reached the central area or the edge for each cluster size, and the average times of earthworm reaction and of the meeting with the centre of the arena.

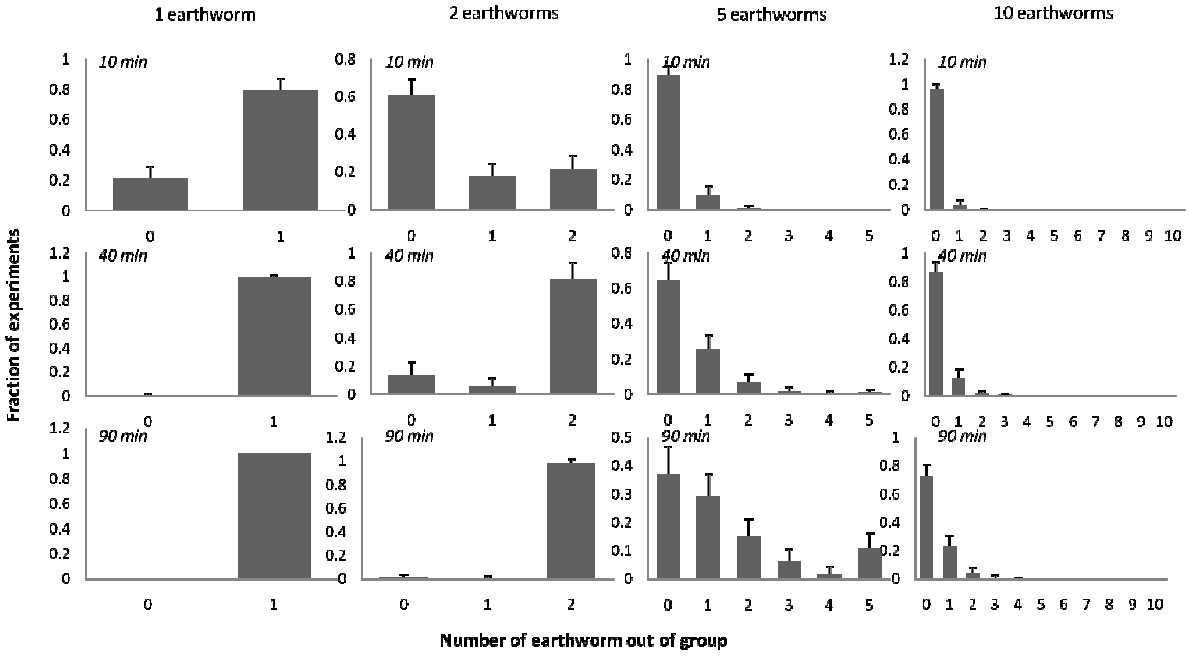
	Experiment number				**Average time (min) ± SD**	
	No choice	Centre of arena	Edge of arena	Total	Earthworm reaction	Center of arena
No earthworm	0	7	39	46	3.5 ± 2.8	7.6 ± 5.0
10 earthworms	1	17	28	46	2.5 ± 2.4	7.2 ± 5.5
15 earthworms	4	22	20	46	3.8 ± 3.6	8.5 ± 7.3

3.2. Assays on the probability of leaving a group

As regards the departure dynamics, our results showed that the fraction of earthworm departure changed over time and was significantly influenced by cluster size. As shown in figure 3a, the probability of leaving a group decreased with the number of earthworms present.



(a)



(b)

Figure 3 – Frequency distributions of observed (a) and expected (b) numbers of individuals leaving the cluster for 1, 2, 5 and 10 earthworms at 10, 40 and 90 min.

The survival curve analysis (Figure 4a) clearly showed that earthworms were less likely to leave the central area when the size of the group increased ($\chi^2_3 = 107$, $p < 0.001$). The period of time needed for the first earthworm to leave the central area depended on cluster size (One

way ANOVA, $F_{3,116} = 54.47$, $p < 0.001$) and this time increased significantly with the cluster size at the beginning of the experiment. Indeed, one earthworm left the central area 11.9 times faster when alone than when it belonged to a group of 10 individuals (Figure 5a).

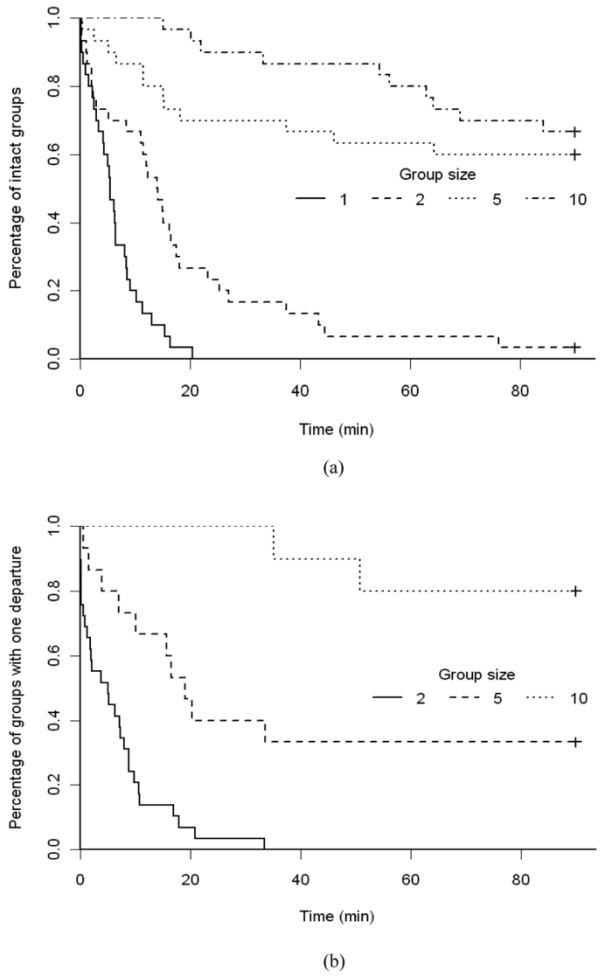


Figure 4 – Results of survival curve analysis for the departure of the first (a) and the second (b) earthworms in each group size (1, 2, 5 or 10 earthworms). The second earthworm departure is seen from the first earthworm departure, so t_0 for the second earthworm is the departure time of the first earthworm

In this context, the survival curve of the intact groups (without any departure) (Figure 4a) was approximated by exponential:

$$F = e^{-at} \qquad (1)$$

where F is the fraction of groups without any departure at time t, and a corresponds to the probability of leaving and is the inverse mean time of the first departure. Due to this approximation, the individual average time of the first earthworm to leave a group (T) can be calculated for each earthworm population (N) with the equation:

$$T = \frac{N}{a} \qquad (2)$$

This function T means that the time of the first earthworm to leave a group increases with the number of conspecifics (N) in the group. Based on figure 6, the equation to express the departure time of the first earthworm in function of the earthworm population size was determined:

$$T = 6.25N^{2.65} \qquad (3)$$

Similarly to the first earthworm, the probability of leaving a group for the second earthworm significantly decreased when the cluster size increased (Figure 4b; $\chi^2_2 = 35.8$, $p < 0.001$) whereas the time to leave increased (Figure 5b; General linear model, $F_{2,38} = 18.0$, $p < 0.001$).

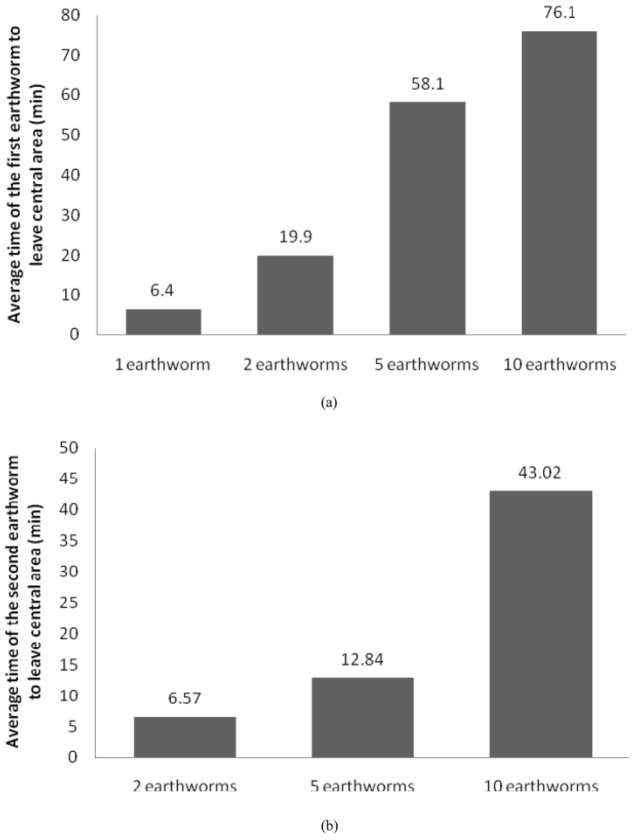


Figure 5 – Average time of the first (a) and the second (b) earthworms leaving central area in the circular arena set-up according to the group size (1, 2, 5 or 10 earthworms). The second earthworm departure is seen from the first earthworm departure, so t_0 for the second earthworm is the departure time of the first earthworm

The second earthworm departure is measured from the first earthworm departure, so t_0 for the second earthworm is the departure time of the first earthworm. Sometimes in groups of 2 earthworms, the second earthworm left the central area by contact with the first earthworm. The second earthworm departure was significantly more rapid when contact between

earthworms was observed, 0.39 min ± 0.68 min (mean ± SD) vs. 8.71 min ± 8.08 min (mean ± SD) when contact was absent (General linear model, $F_{1,27}$ = 20.45, p < 0.001). Due to contact between individuals, the survival curves for the second earthworm leaving the centre of the area can be considered as a double exponential.

$$F = fe^{-bt} + (1-f)e^{-ct} \qquad (4)$$

where f (= 0.24) is the fraction of departure under contact, (1-f) is the fraction of departure contactless, b is departure by contact probability (b = 7.32 min^{-1}) and c (= 0.11 min^{-1}) is the departure contactless probability. With such constant probabilities, the inverse of b and c are equal to corresponding mean delay between the two earthworm departures (with and without contact). For both categories of departure, b-1 and c-1 are close the corresponding mean duration (with contact 0.14 vs. 0.39 min; without contact 9.1 vs. 8.71 min).

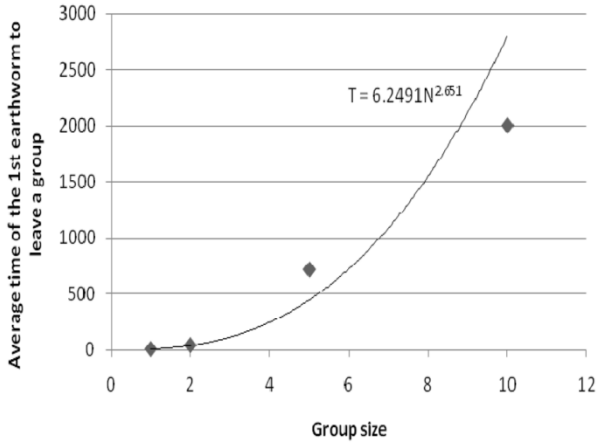


Figure 6 – Time equation of the first departure. Average times of the first earthworm departure in function of the cluster size and its adjustment (black line)

The objectives of the following model were to validate the agreement between our observations at the individual and collective levels and to highlight some characteristics of the collective dynamics such as the emergence of quorums or threshold.

Our data analysis showed that the individual average time of the first earthworm to leave a group increased with the number of conspecifics (N) in the group (see equation 2). The survival curve of the intact groups (without any departure) being approximated by exponential, the individual probability of leaving was therefore the inverse of this average time (see equation 1). We assumed that the probability of leaving was the same for each individual and equal to Q(N), where N is the number of individuals in the earthworms group:

$$Q(N) = \frac{1}{T(N)} \qquad (5)$$

Moreover, for the followers, we neglected the facilitation effect due to the departure of a previous earthworm (see equation 4).

To summarize the model, we assumed a continuous time Markovian jump process, that is, the probability per time unit displaying the response (here, leaving the cluster) is constant over time as long as the stimulus remains the same, here the size of the group, and this probability jumps to a new value when the stimulus changes (the number of individuals within the cluster).

To test the relevance of the parsimonious model and understand the main effects arising from the dynamic fluctuations, Monte Carlo simulations were used where the random aspect of the process is automatically incorporated. The simulations were based on the probability of leaving a group Q(N) previously estimated and being the inverse of mean leaving time. We assumed that each individual obeyed to this function. The different steps can be summarized as follows: (1) initial conditions: the number of individuals within the group (N) was determined at N_0; (2) decision process: two states are possible for each individual within the cluster or outside. At each time step (t), the position of each individual was noted. Then its probability of moving out of the cluster is given by Q(N). Its departure at t depends on the comparison between the calculated value Q and a random number sampled from an uniform distribution between 0 and 1. If its value is less than or equal to Q, the individual leaves the cluster. If not, it stays within it. The probability Q(N) of leaving the group was updated at each simulation step in relation to the number of individuals still present. As in our experiment after having left the cluster, an earthworm will never join the cluster (no entry). Simulation results allowed us to follow the survival of cluster in relation to time and the process was repeated for 90000 steps (180 minutes, a time step = 0.01 min). Monte Carlo simulations were run 6000 times (200 x groups of 30 simulations). The distributions of the numbers of individuals present within the cluster were calculated in relation to time and are compared to the experimental results. There was a good agreement between the theoretical and experimental results for group of 1, 2 and 10 earthworms and it was weaker for groups of 5 earthworms (Figures 3a and 3b). The model showed that the cluster survival follows a quorum with a threshold when the system was considered without entry (Figure 7). Indeed, the proportion of leaving earthworms decreases following a sigmoid curve with the initial size of cluster (N_0). Figure 7 showed that the threshold increased slowly over time. For example, the threshold was about 3.5 earthworms at 60 min and about 5.5 earthworms at 180 min.

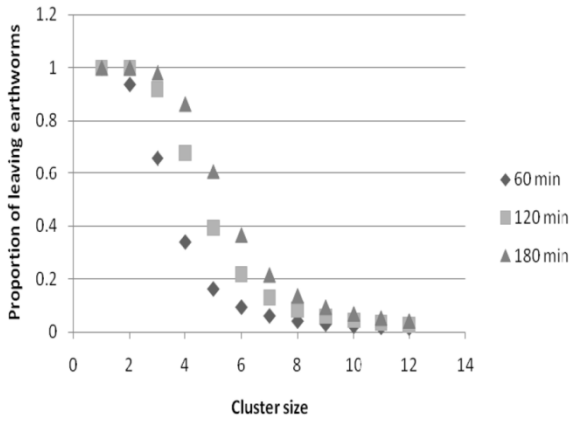


Figure 7 – Critical threshold. Change over time (at 60, 120 and 180 min) of critical threshold according to cluster size

This threshold emerged at the collective level from the dynamics of departure. Indeed, the individual probability of leaving Q(N) did not exhibit any threshold behaviour. Moreover, when the model takes into account both processes – joining and leaving the cluster – it is easy to show that such system exhibits an initial critical size (or threshold) of the cluster (Figure 8a). In this version of the model, we assumed that the cluster is surrounded by a constant population of earthworms and therefore there was a constant probability per time unit (μ) that an earthworm joined the cluster. This probability combined the speed of the earthworms and their surrounding density. In parallel to the joining process, each earthworm may leave the cluster with the probability of moving out of the cluster Q(N).

The simulation started with an initial number of earthworms (N_0) and the time of the simulation was 5h. These long-term simulations showed well the threshold effect.

The sigmoidal shape of the mean population within the clusters as a function of the initial population confirmed the existence of the threshold. This was also confirmed by the distribution of the simulations as a function of the population within a cluster. For a small initial population, a peak was seen at N = 0, showing that most of the clusters collapsed. For a greater initial condition two peaks emerged respectively at N = 0 and for a larger N (Figure 8b). Between these two peaks, there was a minimum corresponding to the threshold. In this case, where the initial value was close to the threshold, some clusters were successful and some other collapse or vegetate. For a large value of N_0 , greater that the threshold, all clusters were successful and grow (Figure 8b). Not surprisingly, this threshold value depended on the probability of joining: lower this probability, greater was the threshold.

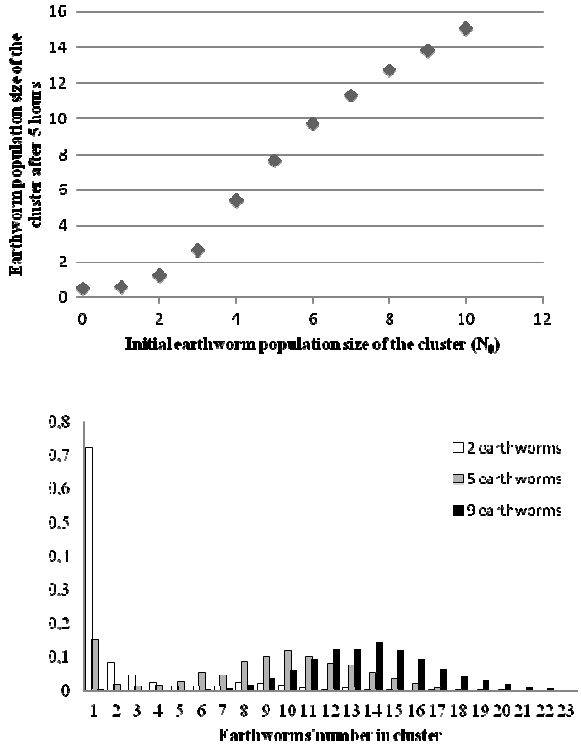


Figure 8 – (a) Mean population within the cluster as a function of the initial population at 5hours with the probability of joining (μ) = 0.02 min^{-1} and $Q(N) = N^{-2.6}/6.25$ min^{-1}. (b) Distribution of the cluster size at 5 hours for three different initial populations: 2 earthworms, 5 earthworms, and 9 earthworms with the probability of joining μ = 0.02 min^{-1} and $Q(N) = N^{-2.6}/6.25$ min^{-1}

4. Discussion

Cooperative effects in *E. fetida* have been demonstrated here leading to the formation of a surprising self-assembled cluster. We furthermore show that olfactory cues are involved in cluster formation and that cluster stability increases with the number of individuals. To the best of our knowledge, no previous studies have identified social structures in earthworms. The two mechanisms governing the cluster formation are the attraction due to olfactory cues and retention by other individuals.

The perception of volatile odorants by *E. fetida* involving some specific corporal receptors associated with neurons could be used to communicate with conspecifics to form aggregates [20]. Many interactions between organisms are based on the emission and perception of volatiles [29]. Until recently, most of the studies on volatile infochemicals were focused on aboveground organisms [30] but odours are also found in the soil and permeate through air-filled pores [29]. These soils include inter-specific interactions between roots plant, soil fungi, nematodes and arthropods. However, no information is available neither on intra-specific interactions nor on earthworms, key components of temperate soil ecosystems. Nevertheless, intra-specific interactions were implicated in pheromone production, inducing thermo-

tolerance in *Tubifex tubifex* and *Enchytraeus albidus*, two aquatic annelids [31]. Moreover, a recent study demonstrates that earthworms are able to use olfactory cues to actively search for food sources [32]. Indeed, earthworms are known to have chemoreceptors, principally on the prostonium or on the buccal epithelium [20].

Surprisingly, the time needed by tested earthworms to choose the cue source was longer than the time needed by tested earthworms that chose as final decision the empty chamber of the Y set-up (12.9 ± 1.1 min vs. 6.7 ± 0.5 min). Some hypotheses can be formulated. Klinotaxis may be one possible explanation for the longer time to choose the cue-associated branch of the olfactometer, as observed in other animal phyla [33]. Indeed, some tested earthworms could perceive a decreased concentration of the signal in the control branch of the olfactometer, turn back and choose the earthworm-connected branch, increasing the mean time observed in finally choosing the cue-associated branch of the olfactometer. An active searching behaviour with stop phases and side-to-side movement like those observed in some insects [34] or nematodes [35] could also explain the longer time period necessary for the earthworms that use chemical cues to locate conspecifics. Some observations during our experiments supported the two hypotheses but further studies will be necessary.

The stability of a formed cluster increases with its size. Similar results were observed with aggregation of cockroaches under shelter [10,36,37]. Jeanson et al. [38] studied aggregation site selection by the ant *Messor barbarous* and found that the probability of ants leaving a selected site decreases with the number of workers at the site. Moreover, an aggregation study on the ant *Lasius niger* showed that the greater the number of ants inside a cluster, the weaker the probability of leaving this cluster [39].

Modelling allows the testing of some rules of interaction based on minimal hypotheses and so helps to verify whether results of simulations follow similar rules of interaction as the observed animal aggregation [7]. Here, good agreement between the theoretical (model) and experimental results confirms that the probability of leaving a group decreases with earthworm number in the cluster. Nevertheless some differences were observed between theoretical and observed distributions of leaving earthworms over time, mainly in a group of 5 earthworms (e.g. after 90 min, 50% of experiments have no departure versus 35% ± 10% (SD) predicted by simulations and 25% of experiments are with 5 departures whereas the simulation predict 11% ± 5% (SD)). These differences can be explained by the role of contacts that we highlighted in groups of 2 and 5 earthworms. These effects of contacts, not included in our model, accelerate the departure of the followers. This hypothesis is enhanced by a previous study where the *E. fetida* earthworms' cooperative capacity to select direction

through contact among individuals has been demonstrated [18]. The model also highlights a threshold in cluster survival and growth when joining and leaving processes were integrated in the model. The value of the threshold depends on the value of joining probability (μ) and a critical threshold was highlighted. However, more detailed studies were necessary to better understand the role of this threshold for example its influence on the spatial organization of the earthworm population.

Cluster formation can bring some advantages by allowing information transfer between individuals [40], promoting cooperation in food source researches [41], facilitating thermo- and hydroregulation [42-44], or increasing protection against predators [45,46]. The enhancement of chemical defences could be one advantage of clustering in earthworms [47]. For the earthworm *E. fetida*, the defensive line comprises cells floating in coelomic fluid and humoral effector proteins secreted into the coelomic fluid. This body fluid is known to contain a variety of cytolytic and bacterial activities to combat potential soil pathogens [48]. *E. fetida* are also the prey of terrestrial flatworms. *E. fetida* secretes yellow coelomic fluid in response to flatworm (*Bipalium adventitium*) attacks, producing an aversive response in the flatworms, and increasing the survival rate of this earthworm species [49]. Such cooperative defence behaviour would be particularly vital when earthworms are under high predation pressure such as *E. fetida*. The resistance to noxious environmental conditions (e.g. flooded soil, dry soil) could be a second advantage of clustering [20]. Aggregation could be the start point to mass migration. Indeed, collective movement in *E. fetida* has been recently reported [18]. Doeksen [50] has observed that individuals of *E. fetida*, which were living in the soil in greenhouse, migrated in large numbers up the sides of buildings during damp, wet and foggy weather. Mass migration has also been observed in earthworms in response to flooded soil conditions [51], but this may now be seen as using these conditions for greater potential gene exchange through active dispersal [52]. In addition to humidity, temperature may be a factor of primary importance in determining the composition and the structure of earthworm communities [53,54]. Control cluster formation could enhance techniques for extraction and sampling of earthworms, for example in vermicomposting. Other behavioural techniques have previously been employed for such purposes, including electrical extraction, and chemical methods extraction [21], but these methods have some inconveniences.

In conclusion, this study provides the first documentation of olfactory cues and conspecifics interaction involved in annelid cluster formation. Several animal species release aggregation pheromones that attract conspecifics for example for optimizing resource use and maintain aggregate [46]. Thus, further elucidation of the specific cues responsible for intra-specific

attraction and of the mechanisms by which earthworms perceive and respond to olfactory cues will enhance our understanding of the ecology of soil ecosystems, in which earthworm play an important role in temperate regions. Furthermore, exploration of earthworm intra-specific behaviour will help us to understand how these animals in group influence soil proprieties.

5. Acknowledgment

We thank the members of the group of E. Haubruge for their continuous support; and D. Conoir and J. Farmakidis for their technical assistances; Ouroboros s.a. society for *E. fetida* earthworms.

Lara Zirbes was financially supported by a PhD grant from the Fonds pour la formation à la Recherche dans l'Industrie et l'Agriculture (FRIA), Belgium.

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4.3. A new case of consensual decision: collective movement in earthworms

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Abstract - Collective movements are reported for many species from microorganisms to humans. But except for a few soil-inhabiting species, intra-specific interactions in soil are poorly studied. Some intra-specific interactions occur in earthworms. Most of them are negative, concerning parameters like the rate of survival, maturation, food ingestion or growth. Virtually nothing is known about collective movement in earthworms that represent the dominant biomass of the soil. This study, the first one on annelids, highlights a consensual decision phenomenon based only on contact between followers. Using an olfactometer set-up and modelling, we show that earthworms *Eisenia fetida* influence each other to select a common direction during their migration. Experiments in a binary choice test showed that contacts between individuals are responsible for collective movement. This coordination in movement could allow earthworms to benefit from forming clusters. The resulting local higher densities, enhancing individual survival and favouring the cooperation, may be at the origin of Allee effects reported for these species.

1. Introduction

Unlike inter-specific interactions (Bonkowski et al. 2000; Salmon & Ponge 2001; Pizl & Novakova 2003; Rasmann et al. 2005), intra-specific interactions in different spheres of soil like drilosphere and detritusphere are less studied, except for ants and termites (Jouquet et al. 2003; Mikheyev & Tschinkel 2004; Tschinkel 2005). Here, we have explored the influence of intra-specific interactions in a particular soil inhabiting group: the earthworms. Indeed, earthworms represent up to 70 per cent of soil biomass (Lee 1985) and are major representatives of drilosphere and detritusphere (Brown et al. 2000). The drilosphere is the soil area directly influenced by earthworms whereas detritusphere corresponds to the litter system at soil surface (Brown et al. 2000). A recent review shows that intra-specific interactions have been studied in 15 lumbricid species. Intra-specific reactions are strongly associated with density and in most cases intra-specific interactions associated with high density have a negative influence on conspecifics like a diminution in rates of growth and maturation, survival or reproduction. Frequently, the growth rate of a population declines with increasing density (Uvarov 2009). Moreover, density seems to be an important parameter to regulate population dynamics in *Eisenia fetida* (Kammenga et al. 2003).

Eisenia fetida belongs to the epigeic earthworms group (Bouché 1977). This ecological category lives on or near the soil surface, typically in the litter layers of forest soils and does not burrow (Lee 1985; Edwards & Bohlen 1996; Römbke et al. 2005). Due to their surface proximity, this ecological category of earthworms is under high predation and so has a short life cycle depending principally on population density and environmental temperature (Lee 1985; Edwards & Bohlen 1996). This cycle life (at 25°C) is characterised by the production of one or more cocoons after mating, 20 days later cocoons hatch and 2 to 10 hatchlings born. These hatchlings become mature (with clitellum) 4 to 6 weeks later (Lee 1985). Epigeics feed on litter and/or the attached microflora and ingest little soil. A few species, best known among them *Eisenia fetida* and *Eisenia andrei*, are specialists in places with high organic matter content (Lee 1985).

Like some other lumbricids, *Eisenia fetida* lives in patchy distribution (Reinecke & Viljoen 1990; Boag et al. 1994; Uvarov 2009). The spatial heterogeneity of the population depends on food distribution and abiotic parameters also on intra-specific interactions, including inter-attraction. Like in other animal species, inter-attraction could generate an Allee effect (Courchamp et al. 2008) and collective movement (Krause & Ruxton 2002). It is recognised that individuals of many species may benefit from the presence of conspecifics. The Allee

effect is defined as a positive relationship between any component of individual fitness and either numbers and density of conspecifics (Stephens et al. 1999)

Collective movement is sometimes observed in earthworm populations. They evade unfavourable environmental conditions like flooded soil and low oxygen tension or seek new habitats (Edwards & Bohlen 1996). Occasionally, the number of earthworms migrating is especially significant (Edwards & Bohlen 1996; Buhl et al. 2006), suggesting that social cues may be responsible for collective movement. Chemical and/or physical 'communication' could occur in earthworms. For example, noxious stimulation, like the electric shock of an earthworm, elicits secretion acting as an alarm pheromone, i.e., an earthworm contacting such secretions escapes more rapidly from the area than from a clean area (Schmidt 1955; Ressler et al. 1968; Jiang et al. 1989). Indeed, metabolites of individual activity perceived by conspecifics acting as alarm pheromones have been reported in different species of earthworms (Ratner & Boice 1971; Rosenkoetter & Boice 1975). *Lumbricus terrestris* may often find its partners by a trail-following behaviour and use contact to choose a sexual partner during the premating (Nuutinen & Butt 1997). While some studies investigate the influence of intra-specific interaction on earthworms' life parameters (survive, growth, reproduction, cocoons hatchability) (Domiguez et al. 1997; Uvarov & Scheu 2005; Uvarov 2009), no research has investigated collective movement in earthworms and the underpinning mechanisms.

In this study, we observed earthworms' behaviour in an olfactometer set-up in order to establish the degree of potential collective movement in *Eisenia fetida*. Further, we investigated whether chemical (trail following) and physical (contact) cues are involved in collective movement in earthworms.

2. Materials and methods

2.1. *Eisenia fetida* rearing

The earthworms (*Eisenia fetida*) used in this study come from vermicomposting Ouroboros s.a. (Belgium). They are reared in PVC boxes (42 cm long, 30 cm wide and 10 cm high) filled with universal compost DCM ®. This compost is changed every two months and cocoons and hatchling earthworms are sorted out in order to be released in a new box with fresh compost. Boxes were kept at a temperature of 23 ± 1°C. For the experiments, only mature earthworms (with a clitellum) were used.

2.2. Binary choice experiments

Binary choice experiments remain one of the easiest ways to study collective decision making and to analyse the relation between individual behaviour and collective decision making (Jeanson & Deneubourg 2009). The distribution of the number of individuals among two alternatives may be different according to the species and situation: it can vary from an equi-distribution to a strong difference between the number of individuals choosing either direction. Two experimental set-ups were used for binary choice experiments: (a.) olfactometer assays and (b.) contact assays.

a. Olfactometer assays

The behaviour of earthworms was tested in a two-arm olfactometer consisting of a central PVC chamber (20 cm x 20 cm x 20 cm) with two equally distributed side arms (9 cm in diameter, 18 cm long) (Fig. 1a). These arms were connected to the central chamber and 25 g of the same food source (sheep manure) was placed in the extremity of each arm. For each experiment the entire system was filled with moist universal compost DCM ® (76 per cent humidity content). An approximately 25g sample of moist compost was dried at 105°C for 48 hours to determine the moisture content (15 repetitions were realized). Forty earthworms, *Eisenia fetida,* were released in the centre of the central chamber. Twenty-four hours after being released, the arms of each olfactometer were disassembled from the central chamber, the compost from each arm was placed in containers and the earthworms were counted. Thirty repetitions were conducted, the olfactometer was washed with distilled water and compost was replaced between each repetition.

b. Contact/ trail assays

A diamond-shaped set-up (15 cm long, 10 cm wide, 5 cm high) with start and end point (3 cm long, 1.5 cm wide, 5 cm high) (Fig. 1b) was used to study contact and trail following for *Eisenia fetida* mature earthworms. This set-up was filled with 56.25 cm^3 of moist universal compost DCM® (76 per cent humidity content). Observations were made under red light in order to not disturb the earthworms (Lee, 1985). This experimental set-up was emptied, washed with distilled water and compost was replaced between each repetition.

In the trail-following assays, two experiments were performed. In the first experiment, one earthworm was placed on the start point set-up and was free to choose a branch. It was then removed when it reached the end point with a food source (2 g of sheep manure). After that, a second earthworm was released at the start point and its choice was observed. This

experiment was repeated 45 times. The two possibilities for earthworms were to move in the same direction or not. In the second experiment, five earthworms (one after the other) were forced to move on the same branch set-up by blocking the other branch with a PVC piece and the choice of the sixth one was recorded. The experiment was repeated 10 times.

In contact trial tests, two earthworms were simultaneously placed at the start point of the set-up and both selected directions were observed and recorded when they reached the end point filled with food resources (2 g of sheep manure). This experimentation was repeated 45 times. Two results could be observed: (1) both earthworms on the same direction (right or left) and (2) one earthworm on the left and one on the right.

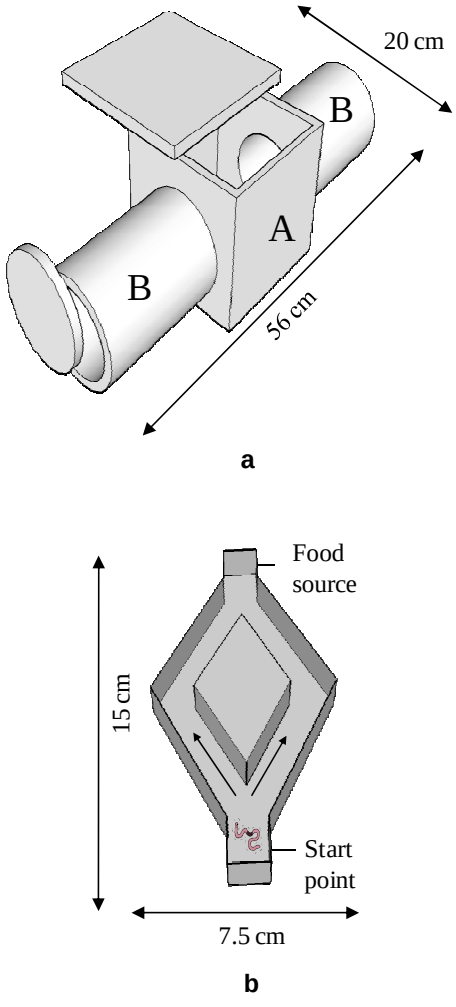


Fig. 1: Experimental set-ups. (a) Two-arm olfactometer to earthworms'cooperation assays. A = central chamber, B = arms of the olfactometer. (b) Behavioural set-up to trail-following and contact bioassays. Start point = point where earthworms were placed at the beginning of the experiments, food source = destination of earthworms

2.3. Statistical analyses

A Chi-square Goodness-of-fit test (Minitab® v15.0, State College, Pennsylvania USA - N = 30, α = 5%, 1 degree of freedom) was used to compare distribution of the number of earthworms in both arms versus the central chamber of the two-arm olfactometer and a binomial distribution.

Statistical differences in direction selection by earthworms were determined by fitting a constant probability generalized linear model to the data. The generalized linear model was used to take into account the non-constant number of earthworms in both arms for the 30 repetitions of the test. Under the assumption of independence of choices of the individual worms, the residual deviance of this model should follow a Chi-square distribution with 29 degrees of freedom (Faraway 2006). An overdispersion test was then realized to check this assumption (R 2.7.1). To graphically display the results of this test, the observed proportions of earthworms on the left branch were reduced by their mean and scaled by their standard deviation, allowing for comparison. Under the hypothesis of independence of movements, this reduced distribution should tend to a reduced normal distribution by Moivre's theorem. So the figure obtained superimposes the density of reduced observed earthworms' proportions in the left arm on the corresponding reduced normal distribution.

For trail-following assays and contact trial tests, a Chi-square Goodness-of-fit test (Minitab® v15.0, State College, Pennsylvania USA - N = 45, α = 5%, 1 degree of freedom) was used to compare theoretical distribution (Respectively, 50%–50% for each possibility) and observed distribution.

2.4. Simulation

Simulation was used to determine whether behaviour observed between two earthworms in contact/trail assays could explain behaviour observed between 40 earthworms in the two-arm olfactometer.

At the start of a simulation, all the individuals (N) are assumed to be in the central chamber. For each simulation, N earthworms were given the choice of leaving the central chamber and choosing between two identical branches. The individual probability of leaving the central chamber (P) was calculated to be 0.84 (see result section for details) using the results of two-arm olfactometer assays. The individual decision of leaving the chamber was checked and depends on the comparison between P and a random number sampled from a uniform

distribution between 0 and 1. If its value is ≤ P, the individual leaves the chamber and its selection of direction is tested. P_L and P_R are respectively the probability of choosing the left or right branch. If the value of a second random number is ≤ P_L the individual chooses the left branch. If not, it takes the opposite direction. For the first individual leaving the chamber, $P_L = P_R = 0.5$ (the two branches of the experimental set-up are identical).

For the others, $P_L = Q$ ($P_R = Q$) if its predecessor chooses the left (right) branch, Q being the probability of following its predecessor and choosing the same direction. Q = 0.5 corresponds to a random choice (no social influence). In case of social interaction, Q > 0.5 corresponds to a social attraction and Q < 0.5 corresponds to the situation where the earthworm prefers to take the opposite direction to its predecessor. The contact trial test where two earthworms were simultaneously placed in the set-up provided the Q experimental value.

The percentage of earthworms that chose the winner branch was counted at the end of the simulation. For both hypotheses (with or without social interaction), the possible outcome of the average from 30 experiments and their distribution was calculated 1000 times.

The simulation was also used to estimate the mean number of earthworms selecting the winner branch as a function of the number of earthworms choosing one direction and of the value of Q.

3. Results

Figure 2a shows the distribution of the total number of earthworms leaving the central chamber of the olfactometer. This distribution was compared to a binomial theoretical distribution which should be observed when no interaction exists between individuals. Among the 1200 tested earthworms (40 earthworms x 30 replicates), 1005 left the central chamber. The theoretical binomial distribution was then generated with an estimated probability of leaving of 0.84 (1005/1200) that an individual leaves the central chamber. In this case, experimental distribution was not different from the theoretical distribution (Chi-square Goodness-of-fit test, $\chi^2_4 = 4.09$, P = 0.394). The results were not biased by experimental set-up: at the end of 30 repetitions, the number of earthworms was not significantly different in both arms of the olfactometer (right = 521, left = 484).

The generalized linear model showed that the distribution of the choice of one arm (number on the left arm) was significantly different (Deviance test, $\chi^2_{29} = 71.05$, P < 0.001) from the binomial distribution with a trend toward statistical overdispersion. These results strongly imply dependence between the choices of earthworms (Fig. 2b). These results show that

earthworms moving out of the central chamber influence the directional choice of other earthworms, but not their propensity to leave the central chamber.

To determine whether earthworms follow a conspecific trail, two behavioural trial tests were used. In the first trail-following test, one *Eisenia fetida* (N = 45) earthworm was placed at the starting point and was free to choose a direction (right or left). In this assay, 46.7 per cent of the earthworms chose the same direction as the previous one and 53.3 per cent did not. The results were not significantly different from 50%–50% (Chi-square Goodness-of-fit test, χ^2_1 = 0.2, P = 0,655). The trail-following test was conducted in the same conditions as previously. However, five *Eisenia fetida* earthworms (N = 10) were forced to move in the same direction (right or left) and the direction of the sixth earthworm was recorded. In this case, five earthworms followed their predecessors and five earthworms did not. These results indicate that the earthworms did not follow a conspecific trail by olfactory orientation.

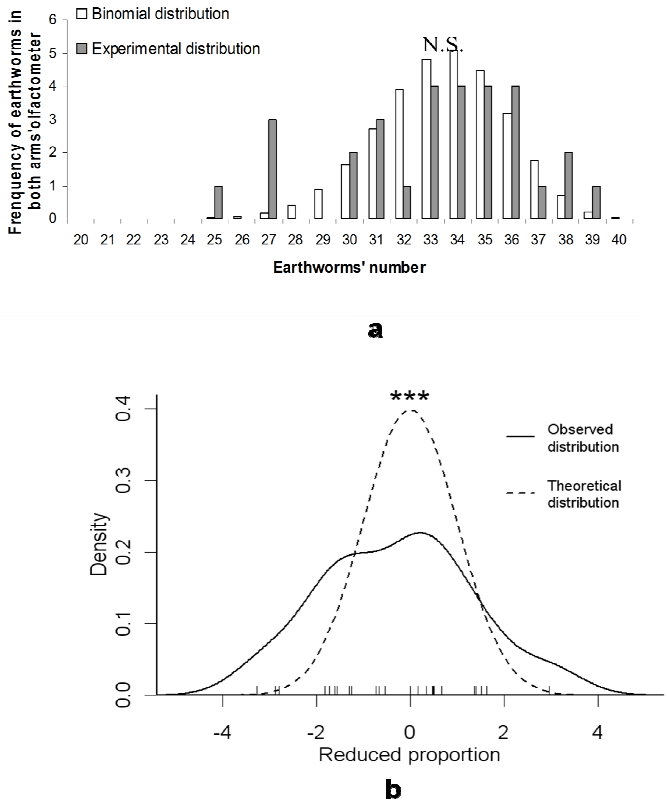


Fig. 2: Experimental results. (a.) Observed (grey) and theoretical (white) distributions of *Eisenia fetida* earthworms in both olfactometer arms. N.S. (Not Significant) indicates no significant difference between observed and expected distribution. (b.) The comparison of theoretical (discontinuous stroke) and experimental distributions (continuous stroke) of the proportion of earthworms on the left olfactometer arm shows an excess of off-center proportions which indicate non independent choice in earthworms' movements. Bars at the bottom of the chart represent reduced observed proportions that were used to calculate the density of probability. Triple asterisk indicates a significant difference between observed and expected distributions at $p < 0.001$.

To establish whether or not contact between *Eisenia fetida* earthworms influences *Eisenia fetida* interaction, two earthworms (N = 45) were simultaneously placed at the starting point (Fig. 1b). In this experiment 69 per cent moved together while 31 per cent of the pairs did not select the same direction. There was a significant difference (Chi-square Goodness-of-fit test, $\chi^2_1 = 7.53$, P = 0.006) between the experimental and theoretical (i.e. absence of any interaction) distributions (Fig. 3). The contact between earthworms strongly influences the directional choice of the follower. Indeed, the probability of following the predecessor (Q) is estimated to be 0.7.

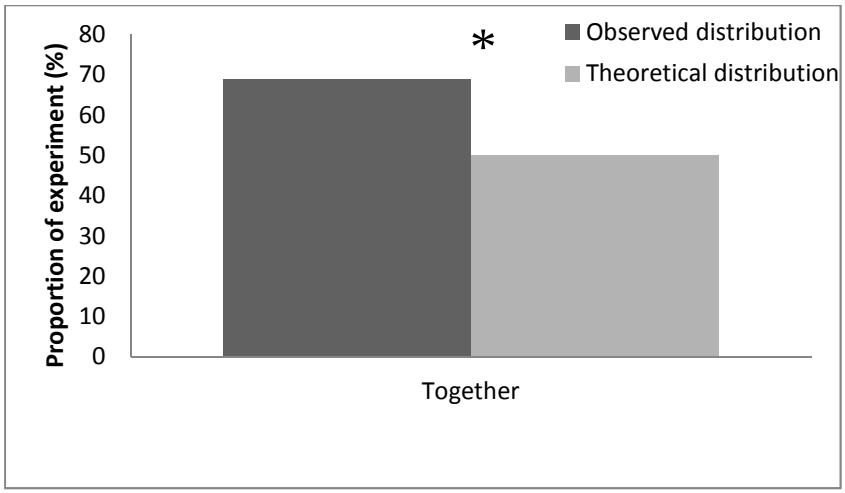
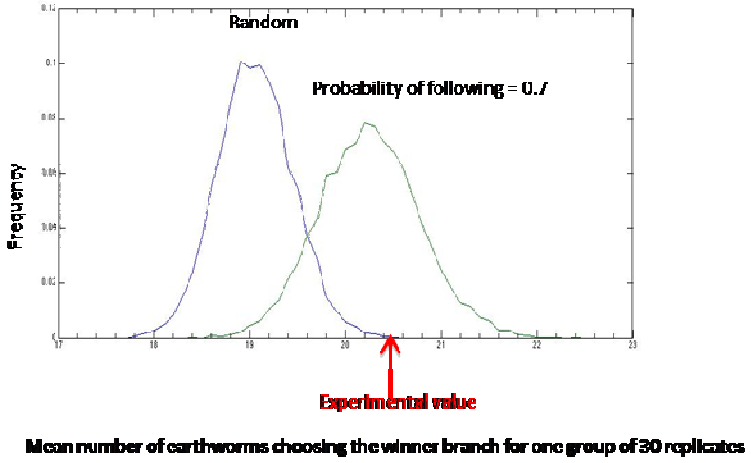


Fig. 3: Observed (dark grey) and theoretical (light grey) distributions of the proportion of earthworms which select a direction together or not in behavioural bioassays when two earthworms were in contact at the start of the experiment. *Significant difference between observed and theoretical distributions at $p < 0.05$.

A simple model shows that such probability of following generates the earthworm distribution among both branches when groups of 40 individuals were tested in the two-arm olfactometer. The number of earthworms selecting the winner branch was counted at the end of the simulation. The mean distribution of 30 experiments average was calculated (Fig. 4a). The probability of obtaining an equivalent average number of earthworms on the chosen branch from experimental data (mean = 20.27) was maximal with the Q value resulting from contact trial tests (Q = 0.7). With weaker social interaction (Q < 0.6) the probability of obtaining an equivalent average number of earthworms on the chosen branch from experimental data was < 0.05 and without social interaction the probability was slight (= 0.002). Our simulation has shown that contact explains collective movement observed between *Eisenia fetida* earthworms. We have also shown that the average fraction of earthworms on the winner

branch decreases as the number of earthworms increases for each Q value (Fig. 4b) (See supplementary material for equation development.). This diminution was abrupt for groups of 15 earthworms, but was more gradual for larger number of tested earthworms. So the higher the number of tested earthworms in the olfactometer, the greater the symmetry between the two olfactometer arms. These results highlight the fact that the mechanism worked best for small numbers of earthworms.



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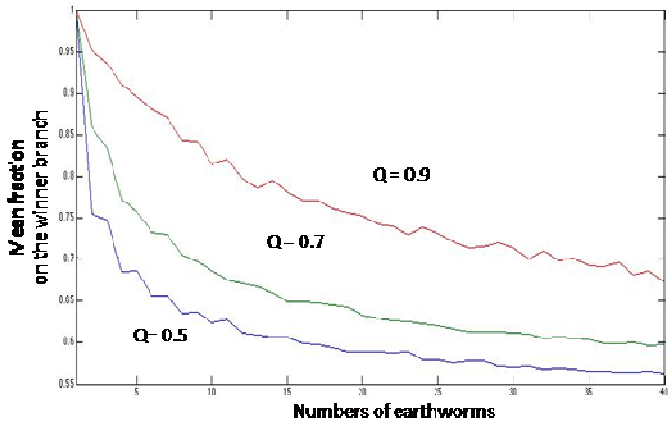

b

Fig. 3: Simulation results. (a.) Results for parameter values P = 0.84, Q = 0.5 and Q = 0.7. These distributions of the mean number of earthworms in the winner branch of the two-arm olfactometer are generated after 1000 runs of 30 replications. (b.) The curves show the average number of earthworms in the winner branch for different earthworm numbers and for different probabilities to follow its predecessor. In this case, the probability to leave the central chamber was equal to 1 (p = 1).

4. Discussion

Individual earthworm propensity to leave the central chamber was not dependent upon the number of earthworm that already left the central chamber. However, the experimental distribution (see Fig. 2a) was slightly bimodal due to peaks at 25 and 27, suggesting that the earthworm probability of leaving the central chamber was weakly influenced by the departure of conspecifics (Sempo et al. 2009).

We have demonstrated the *Eisenia fetida* earthworms' cooperative capacity to select direction through contact among individuals. To our knowledge this is the first example of collective orientation in animals based only on contact between followers and also the first one of collective movement in annelids. It is well known that earthworms use tactile receptors on the surface body for thigmotactic responses (Lee 1985). Moreover, earthworms have chemoreceptors principally on the prostonium or on the buccal epithelium (Edwards & Bohlen 1996). Contact with surface secretion of earthworms has a quieting influence on other earthworms of the same species, i.e., when one or more earthworms settled down anywhere the others stopped more readily in that place (Loeb 1973). Earthworms *Eisenia fetida* were able to perceived pheromone, such as alarm pheromone (Jiang et al. 1989; Ressler et al. 1968; Rosenkoetter & Boice 1975), however pheromone following did not seem to be a mechanism used by *Eisenia fetida* to move together. Trail following could have an effect on earthworms behaviour if the number of earthworms forced to move in the same direction was increased. This possibility should be explored further in future experimental research. Nevertheless, it is unlikely that, in natural environment, more than five earthworms follow. Collective movement mediated by chemical and/or physical contact has been observed in a large diversity of invertebrates (Costa 2006; Simpson et al. 2006; Huang et al. 2007). *Eisenia fetida* seems to use the contact strategy to collectively move with conspecifics. In our experimental conditions, only the predecessor influenced the follower choice. So any amplification process was observed. However, we did not exclude an amplification process if the flow of earthworms increased.

Intra-specific interactions have been observed in some other ecological group of earthworms. Positive interactions exist between hatchlings and conspecific adults during the development in anecic species (Lowe & Butt 2002; Grigoropoulou et al. 2008). Physical contacts occur and influence the burrowing behaviour in anecic species, but not in endogeic species (Capowiez 2000). Our observations showed that intra-specific interactions were also present in epigeic species. But some future experiments are necessary to confirm collective movement in other ecological categories.

The collective movement could also be a mechanism leading earthworms to cluster. The enhancement of chemical defences could be one advantage of clustering (Wertheim et al. 2005). For the earthworm *Eisenia fetida*, the defensive line comprises cells floating in coelomic fluid and humoral effector proteins secreted into the coelomic fluid. This body fluid is known to contain a variety of cytolytic and bacterial activities to combat potential soil pathogens (Bruhn et al. 2006). *Eisenia fetida* are also the prey of terrestrial flatworms. *Eisenia fetida* secretes yellow coelomic fluid in response to flatworm, *Bipalium adventitium*, attack producing an aversive response by the flatworm thus increasing the survival rate of this earthworm species (Fiore et al. 2004). Aggregations of earthworms could produce greater amounts of defensive compounds thus providing better resistance to soil pathogens and predators. Such cooperative defence behaviour would be particularly vital when only some earthworms are under high predation pressure. The resistance to bad environmental conditions (flooded soil, dry soil …) could be a second advantage of clustering (Edwards and Bohlen, 1996).

These results strongly suggest a positive relationship between individual fitness and density and may explain that an Allee effect is present in earthworms (Courchamp et al. 2008).

5. Acknowledgements

We thank the staff member of the Functional and Evolutionary Entomology Unity for their continual support; D. Conoir for his technical assistance; and the Ouroboros s.a. society for *Eisenia fetida* earthworms.

We are grateful to Gary Felton (Pennsylvania State University) for his valuable comments on the manuscript.

Lara Zirbes was financially supported by a PhD grant from the Fonds pour la formation à la Recherche dans l'Industrie et l'Agriculture (FRIA), Belgium.

6. Authors' contributions

L.Z. conceptually designed and coordinated all experimental works, conducted most measurement and wrote the manuscript. E.H. has supervised the study. J.L.D. and Y.B. made all modelling, simulation and statistical analyses.

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8. Supporting Information

Additional Supporting Information may be found in the online version of this article: Data 1. Equation to describe earthworms; interaction.

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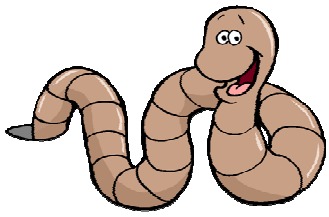
Chapter 5: Conclusions, discussion and perspectives



Vermicomposting is a recently developed biological technique used to minimize environmental pollution (Lee, 1985; Dominguez, 2004); however, the process currently involves some inconvenient elements, including the need to sample the incipient earthworm population before packaging the end product (vermicompost). In our region, *Eisenia fetida* are an earthworm species commonly used in vermicomposting processes (Edwards, 2004). Gaining a better understanding of the behaviour of *E. fetida*, will help us to find a solution to improve the efficiency of earthworm sampling during vermicomposting. It is within this context that the research outlined this thesis was conducted. We wanted to explore food foraging strategy and intra-specific behaviour in *E. fetida*, including the use of chemical communication, in order to develop a behavioural technique for earthworm extraction.

In the context of food foraging behaviour, we have clearly demonstrated that *E. fetida* are attracted by olfactory cues associated with *G. candidum*, a soil fungi, and our research thereby corroborates previous reports that earthworms are able to actively search for a food source. Moreover, we identified for the first time two specific volatile compounds from the filtrate of *G. candidum* colonies implicated in *E. fetida* attraction, the esters ethyl pentanoate and ethyl hexanoate. Chemotaxis to at least 50 volatile compounds was observed in *C. elegans*, and specific neurons and genes implicated in this behaviour have been described (de Bono et al., 2002). The perception of volatile compounds by *E. fetida* may also involve some specific corporal receptors associated with neurons. Indeed, earthworms are known to have chemoreceptors, principally on the prostonium or on the buccal epithelium that are associated with the nervous system and more particularly with axons and dendrites (Edwards and Bohlen, 1996). The foraging strategy of *E. fetida* may also bear similarity to social strains of *C. elegans*, as these worms have been observed to aggregate in areas where bacteria are numerous (de Bono and Bargmann, 1998). Furthermore, exploration of earthworm olfaction will help us to understand how these animals orient themselves and coordinate their behaviour. For example, there is some evidence that *L. terrestris* follows mucus trails to find its partner, but the role of volatile perception in such interactions is yet to be documented.

Chemical interactions between epigeic earthworms and microorganisms have potential applications in developing new behavioural techniques, for example to extract and sample earthworms in vermicomposting. Indeed, different behavioural techniques have already been used to sample earthworms, including heat extraction, electrical extraction, mechanical vibration, trapping or mark-recapture, and more pertinently, chemical extraction with a natural repellent or chemical irritant like formalin, mustard extract, or exotic plant extract.

The two esters presented above as provoking attraction activity in earthworms could be added this final category of extraction, but using a technique based on attraction rather than on repulsion. However, further research is necessary to test the efficiency of the extraction in the context of an industrial vermicomposting process, and to verify whether the two esters present a toxicity risk for plant grown in the resulting vermicompost. A simple test could clarify these two particular points. The cost of such sampling method could also be considered. Earthworm sampling with fungi filtrate could also be industrialised in vermicomposting processes. This method presents some advantages, presented as a water soluble solution easy to disperse on the vermicompost surface, with no toxic compounds for earthworms and a low production cost. However the impact on plant growth of filtrate residue in vermicompost could be further investigated.

Viewed from another angle, our research discoveries can be seen as the first step in the "domestication" of the earthworm; we have demonstrated that we can modify the natural movement of *E. fetida*. This species belongs to the epigeic ecological category, lives above the mineral soil surface (typically in the litter), does not burrow, and moves principally horizontally. In our experiment, we showed that this species of earthworm was able to move vertically (up to distance of one meter) only when a filtrate of *G. candidum* colonies was present. With further experiments we could provide an "earthworm solution" to concentrate earthworm populations (not only epigeics but also anecics and endogeics) in soil or gardens, thereby improving soil quality through earthworm activities including, for example, making available nutrients for plant production, soil and organic matter transport, soil aeration by building burrows, and seed relocation.

In an intra-specific context, experiments using a 2-arm olfactometer have demonstrated that contacts between individuals are implicated in the collective movement of *E. fetida*. Contact with surface secretion of earthworms has a quieting influence on other earthworms of the same species, i.e., when one or more earthworms settled down anywhere the others stopped more readily in that place. Collective movement mediated by chemical and/or physical contact has been observed in a large diversity of invertebrates. This coordinated behaviour allows earthworms to locate food sources more efficiently and could also be a mechanism leading to cluster formation to enhance chemical defence. Indeed, in rearing conditions, we have observed earthworm groups and unexpected self-assembled *E. fetida* clusters. For the first time, we documented the ability of *Eisenia fetida* to join and leave a cluster, and clarified the mechanisms of cluster formation for the compost earthworm. The

two mechanisms governing the cluster formation are 1) attraction due to olfactory cues, and 2) retention by other individuals. Collective movement and cluster formation can bring some advantages by allowing information transfer between individuals, promoting cooperation in the search for food sources, facilitating thermo- and hydro-regulation, or increasing protection against predators. Surprisingly, these results suggest a positive interaction between individual fitness and population density and may indicate that an Allee effect is present in earthworms, in contradiction to previous studies showing negative density dependence for example (for rates of growth, survival, reproduction, etc…). These new results modify the current view of earthworms as animals lacking in social behaviour. These results are not directly related to vermicomposting, but control of collective movement and cluster formation could enhance techniques for extraction and sampling of earthworms. We can envisage that with further extrapolation if this research we could begin to control earthworm movement, compelling them to regroup in a chosen place where they could be easily and rapidly collected

In these two sections of the thesis, chemical ecology was an excellent approach in our study of earthworms. To our knowledge, no previous study has explored chemical communication in earthworms, although some studies have shown chemical interaction in soil concerning other organisms, such as nematodes (Boff et al., 2001; Hong and Sommer, 2006). In one such case, an insect-induced belowground plant signal, (E)-ß-caryophyllene, which strongly attracts an entomopathogenic nematode, was identified using a 6-arm olfactometer (Rasmann et al., 2005). Some researchers have suggested that an olfactometer could also be used to study earthworm responses to plant signals, trophic earthworm-rhizosphere interactions, and others earthworm behaviours (Curry, 2007). In this thesis, olfactometer devices have revealed the attraction of earthworms to a food source (filtrate of *G. candidum* colonies) and congeners. Chemical analyses of volatile compounds emitted by the food source show two specific esters implicated in earthworms attraction. Similar analyses could be used to identify volatile compounds emitted by earthworm groups, which could then be tested individually using an olfactometer to find which compound(s) is (are) implicated in conspecific interactions.

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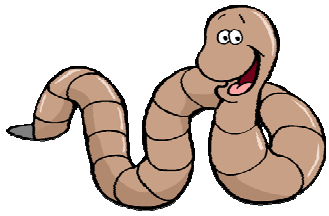
Chapter 6: List of publications, oral presentations and posters



6.1. Publications

Lara Zirbes, Caroline Collin, Joseph Dufey, Pham Khanh Tu, Nguyen Duyet, Fréderic Francis, Philippe Lebailly, Eric Haubruge, et Yves Brostaux (2009) Mise en relation de la diversité des vers de terre et des caractéristiques du sol de Thua Thien Hue (Centre Vietnam). Tropical Conservation Science, 2(3): 282-298.

Zirbes Lara et Haubruge Eric (2009) Les vers de terre: écologie et gestion des déchets. Le Canard Déchaîné du Kauwberg.

Lara Zirbes, Jean-Louis Deneubourg, Yves Brostaux & Eric Haubruge (2010) A new case of consensual decision: collective movement in earthworms. Ethology, 116: 546-553.

Lara Zirbes, Quentin Renard, Joseph Dufey, Pham Khanh Tu, Hoang Nghia Duyet, Philippe Lebailly, Frédéric Francis, Eric Haubruge (2011) Valorisation of a water hyacinth in vermicomposting using an epigeic earthworm *Perionyx excavatus* in Central Vietnam. Biotechnology Agronomy Society and Environment, 15(1): 85-93.

Lara Zirbes, Mark Mescher, Véronique Vrancken, Jean-Paul Wathelet, François J. Verheggen, Philippe Thonart, Eric Haubruge (2011) Earthworms use odor cues to locate and feed on microorganisms in soil. Plos One, 6(7): e21927.

Lara Zirbes, Philippe Thonart, and Eric Haubruge (2011) Microscale interactions between earthworms and microorganisms. Biotechnology Agronomy Society and Environment, (accepted)

Lara Zirbes, Yves Brostaux, Mark Mescher, Maxime Jason, Eric Haubruge, Jean-Louis Deneubourg (2011) Self-assemblage and quorum in earthworms *Eisenia fetida* (Oligochaete, Lumbricidae) Plos One (under review)

6.2. Oral presentation

L. Zirbes, J-L Deneubourg, E. Haubruge (2008) Social behaviour in *Eisenia fetida* (Oligochaeta, Lumbricidae). 15th Benelux Congress of Zoology, Liège (Belgium), 30-31 octobre 2008.

Lara Zirbes, Caroline Collin, Joseph E. Dufey, Pham Khanh Tu, Hoang Nghia Duyet, Philippe Lebailly et Eric Haubruge (2009) Diversité des lombriciens dans les sols de la

zone côtière sableuse de la province de Thua Thien Hue (Vietnam). Improving food crop productivity in the coastal sandy area of the Thua Thien Hue province central Vietnam, Hué (Vietnam), avril 2009.

Zirbes Lara, Thonart Philippe, Wathelet Jean-Paul, Haubruge Eric (2010) Interaction between earthworms and soil fungi: volatiles attraction. The 9th International Symposium on Earthworm Ecology, Xalapa (Mexico), 5-10 septembre 2010.

Zirbes L., Deneubourg J-L., Brostaux, Y., Haubruge E. (2010) Cluster formation in *Eisenia fetida* (Oligochaeta, Lumbricidae). 17th Benelux Congress of Zoology, Ghent (Belgium), 22-23 octobre 2010.

6.3. Posters

Lara Zirbes, Philippe Thonart, Jean-Paul Wathelet, Eric Haubruge (2010) Chemical communication between earthworms and soil fungi. Journée scientifique annuelle de la Société Royale de Chimie: Chimie verte, Gembloux (Belgium), 14 octobre 2010.

Zirbes, Lara; Verheggen, François J.; Mescher, Mark; Wathelet, Jean-Paul; Thonart, Philippe; Haubruge, Eric (2011) Earthworms smell microorganisms in soil. International Symposium of Chemical Ecology, 27th Annual Meeting, British Columbia (Canada), 24-28 Juillet 2011